EFFECT OF THE COMPOSITION OF THE DIET ON THE RIBOFLAVIN REQUIREMENT OF THE RAT[1]

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In a previous report (Mannering, Lipton and Elvehjem, '41) data were presented to show that the fat content of the diet had a marked effect on the riboflavin requirement of the rat. The feeding of riboflavin-low dextrin rations in which a large proportion of the carbohydrate had been isocalorically replaced by fat increased the amount of riboflavin needed by rats for growth and enabled the production of deficiency symptoms of greater severity than are usually observed. Animals fed these high fat rations developed a spastic paralysis of the hind quarters which could be prevented or cured by the administration of riboflavin. Shaw and Phillips ('41) have substantiated the above work and have been able to correlate the paralysis with degenerative neural changes.

The increased riboflavin requirement resulting from the substitution of fat for dextrin might be explained on the basis that an increased cellular demand for the vitamin occurs during the metabolism of fat, or, as seems more likely from the experiments that are to follow, that such an alteration in dietary constitution results in a decreased synthesis of available riboflavin by intestinal bacteria. Several papers have appeared in the literature emphasizing the role of dextrin in promoting the intestinal synthesis of certain vitamins. The coprophagy studies of Guerrant, Dutcher and Tomey ('35) and Guerrant, Dutcher and Brown ('37) demonstrated that the feces of dextrin-fed rats contained greater quantities of undifferentiated B complex than the feces from rats fed sucrose, lactose, glucose, or cornstarch. Guerrant and Dutcher ('34) studied the effects of various dietary constituents on the requirement of rats for thiamine and vitamin G (the heat stable portion of the

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B complex). They concluded that fat and protein had no effect, that higher levels of salts increased, and that agar, cellulose and dextrin decreased the amount of vitamin G needed in the diet. Although riboflavin was not considered independently of vitamin G, these studies strongly suggest that the amount of riboflavin required in the diet by the rat may depend in part on the amount of riboflavin supplied from an enteric source, and that this, in turn, can be greatly influenced by certain dietary ingredients, particularly dextrinized cornstarch.

The present investigation was undertaken to obtain further information regarding the relationship between dietary fat, dextrin, and riboflavin, and was later extended to include a study of the effect of other carbohydrates and of fat on the requirement for and fecal excretion of riboflavin.

EXPERIMENTAL AND RESULTS

Effect of substituting fat for dextrin

The composition of the riboflavin-low diets used is presented in table 1. Rations K-25 and K-27, containing 25 and 40% of fat, respectively, are modifications of high carbohydrate ration K-24 made by isodynamically replacing dextrin with lard. Ration K-26 differs from ration K-25 only in that a fat of vegetable origin [3] was substituted for lard. All rations used in this series and those following were kept under refrigeration. The dextrin was prepared by making a paste of commercial cornstarch and water, autoclaving for 3 hours at 15 pounds pressure, drying, and grinding.

Three-week-old male albino rats weighing 35 to 40 gm. were partially depleted of their riboflavin reserves for 2 weeks on ration K-24 before being divided into four groups of twelve animals receiving the above diets and varying amounts of riboflavin (0, 3, 6, 9, and 12 μg. per day). The riboflavin was fed in ½ ml. of a 25% ethanol solution which was pipetted into small dishes. The animals were housed in individual cages having ½ inch mesh screen bottoms. Food consumption records were kept throughout the supplementation period. Spilled food was collected on paper towels placed beneath the cages and weighed. The results obtained during the 7 weeks following the depletion period are summarized in table 2.

Examination of the table reveals that at each level of riboflavin intake the growth of the animals receiving the high dextrin ration (7% fat) was superior to that of rats fed the 40% fat diet. The growth of animals fed the 25% fat rations was intermediate. No important dif-

[3] Crisco.

TABLE 1
Riboflavin-low rations.

CONSTITUENTS	K-24	K-25	K-26	K-27	K-28	K-29	K-30*	K-31*	K-32*	K-33*	K-34*	K-35*	K-36*	K-37*	K-38*	K-39*
Dextrin, gm.	71	37.5	37.5	21.5					73	22.5						
Sucrose, gm.					71	21.5	73	22.5			33		73	22.5		
Lactose, gm.											40	22.5				
Cornstarch, gm.															73	
Extracted dextrin,[1] gm.																73
Cellulose,[2] gm.													10	10		
Lard, gm.		14		22		22		22.5		22.5		22.5		22.5		
Crisco, gm.			14													
Corn oil, gm.	2	2	2	2	2	2	5	5	5	5	5	5	5	5	5	5
Washed butterfat,[3] gm.	3	3	3	3	3	3										
Cod liver oil, gm.	2	2	2	2	2	2										
Casein,[4] gm.	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
Salts IV,[5] gm.	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Liver extract[6]	(4)	(4)	(4)	(4)	(4)	(4)										
Thiamine, mg.	.3	.3	.3	.3	.3	.3										
Pyridoxine, mg.	.4	.4	.4	.4	.4	.4										
Pantothenic acid, mg.	.5	.5	.5	.5	.5	.5										
Nicotinic acid, mg.	5	5	5	5	5	5										
Choline, mg.	100	100	100	100	100	100										

All rats fed the above rations received 2 drops of haliver oil per week.

[1] Extracted continuously for 3 days with hot 95% ethanol.

[2] Cell-U flour.

[3] Washed 3 times with hot water.

[4] Borden's purified casein, supplied under the trade name "Labco".

[5] Hegsted et al. ('41).

[6] Riboflavin-free liver extract (Wagner et al., '40). Mixed into the ration in amounts equivalent to 4% of the original liver powder.

* Rats receiving these rations were given a daily oral supplement of the following vitamins (μg.) dissolved in ½ ml. of a 25% ethanol solution: thiamine, 40; pyridoxine, 50; pantothenic acid, 150; nicotinic acid, 500; and choline, 10,000.

ferences could be shown between the groups receiving lard and Crisco. If the gains in weight of the rats fed each of the rations are plotted against the daily riboflavin dosage (omitted here to conserve space), it can be shown that increasing the fat in the diet to 40% at the expense of dextrin had an effect of increasing the riboflavin requirement about 4 to 6 μg. per day. Similarly, the feeding of rations containing 25% fat increased the requirement by about 2 μg. per day. The economy of food utilization, calculated as grams gained per 100 Cal. consumed, is closely correlated with the responses in growth made by the rats receiving the different rations and varying amounts of riboflavin.

TABLE 2

Growth of rats receiving rations containing different percentages of fat and graded amounts of riboflavin. (Each figure represents the average response of 3 rats.)

RATION NO.	TYPE OF RATION	DAILY RIBOFLAVIN INTAKE IN MICROGRAMS	GRAMS GAINED IN 7 WEEKS [1]	GRAMS GAINED PER CALORIE FED × 100 (7 WEEKS)
K-24	High dextrin	0	12	1.1
		3	72	5.7
		6	102	6.9
		9	148	7.1
		12	170	8.0
	Average		101	5.8
K-25	25% lard	0	10	.7
		3	41	4.0
		6	71	5.4
		9	88	6.6
		12	121	7.1
	Average		66	4.8
K-26	25% Crisco	0	8	.9
		3	39	4.1
		6	61	5.7
		9	98	6.6
		12	116	6.6
	Average		65	4.8
K-27	40% lard	0	4	.4
		3	16	2.1
		6	53	4.8
		9	84	5.9
		12	98	6.9
	Average		51	4.1

[1] Total grams gained during 7 weeks following 2-week depletion period on riboflavin low ration K-24.

That a 40% level of fat in the diet does not restrict the growth of rats when adequate riboflavin is supplied was demonstrated by raising two groups of four weanling male rats on rations K-24 and K-27 plus 100 μg. of riboflavin per day. At the end of 6 weeks the animals fed the high dextrin ration showed an average weight increase of 184 gm. while those receiving the high fat diet had gained an average of 189 gm.

It was conceivable that in riboflavin deficient rats the deleterious effect of fat might be due to an impairment of fat absorption. In order to test this possibility fat balance studies were conducted during the third and fourth weeks following the depletion period on rats receiving the high dextrin (K-24) and high fat (K-27) rations. Fat retention was excellent in all animals regardless of the type of ration fed or of the riboflavin intake. The average retention of the fat of ration K-24 was 96% (95 to 99) and of ration K-27, 98% (97 to 99).

The total fat content of the livers of all the rats was determined in an attempt to detect a disturbance in fat metabolism or transport. Liver fat on a dry basis varied between 1.6 and 5.9%. The variations could not be associated with either the amount of riboflavin or the type of ration fed.

Effect of substituting fat for sucrose

As mentioned previously, the effect of dietary fat in increasing the riboflavin requirement might be due not to the fat itself but to the replacement of dextrin, a carbohydrate known to stimulate the synthesis of vitamins by intestinal bacteria. Sucrose apparently lacks a similar stimulatory property. If the problem is essentially one of bacterial synthesis, the use of sucrose rather than dextrin as the source of carbohydrates should reduce growth, and the substitution of fat for sucrose should not result in a further reduction. With this in mind the following experiment was designed.

Groups of sixteen male weanling rats were partially depleted of their riboflavin reserves by feeding for 2 weeks the following riboflavin-low rations (table 1): K-24 (high dextrin), K-27 (40% fat, dextrin), K-28 (high sucrose), and K-29 (40% fat, sucrose). The animals within each group were then given varying amounts of riboflavin, namely, 0, 6, 12, 24, and 36 μg. per day. The growth responses of the rats during the 6-week experimental period including the first 2 weeks of depletion are given in table 3.

The superiority in growth of rats receiving the high dextrin ration over animals fed the dextrin-lard ration is again substantiated. This same relationship cannot be shown when sucrose is employed as the

source of carbohydrate. This is in agreement with the observations of Potter, Axelrod and Elvehjem ('42) who were unable to show any effect of fat on the riboflavin requirement of dogs fed sucrose rations. The growth of rats fed the high sucrose ration was not significantly greater than that of animals fed either of the high fat diets. Rats fed the lard-dextrin ration, K-27, grew no better than those receiving the sucrose or lard-sucrose diets although this diet contains considerable amounts of dextrin. It would appear that fat reduces or eliminates the riboflavin sparing effect of dextrin when these two constituents are included in the same ration.

TABLE 3

Growth of rats receiving different riboflavin-low diets and various levels of riboflavin. (Total grams gained in 6 weeks, including 2-week depletion period.)

RATION NO.	TYPE OF RATION	MICROGRAMS OF RIBOFLAVIN/DAY [1]				
		0 (4)	6 (3)	12 (3)	24 (3)	36 (3)
K-24	Dextrin	33	69	88	159	159
K-27	Lard-dextrin	4	40	75	116	124
K-28	Sucrose	21	43	89	121	149
K-29	Lard-sucrose	12	46	75	128	153

[1] Number of rats indicated by numbers in parentheses.

The comparison of the sucrose and high-fat sucrose rations was repeated by raising two groups of five young male rats on rations K-28 and K-29 plus a suboptimal daily dose of 6 μg. of riboflavin. The preliminary depletion period was omitted. Food consumption records were kept throughout the 7-week experimental period. The rats fed the high-sucrose ration gained an average of 82 gm. and those receiving the 40% fat-sucrose diet showed a weight increase of 76 gm. The utilization of the two rations was comparable, 5.9 and 5.4 gm. gained per 100 Cal. consumed of rations K-28 and K-29, respectively.

Effect of various carbohydrates and of fat on the requirement for and fecal excretion of riboflavin

The above work suggests that the beneficial effect of dextrin is due to its stimulation of the production of available riboflavin by the intestinal flora of the rat. If this concept is correct, a positive correlation might be found between growth and the amount of riboflavin excreted in the feces or contained in the cecum. The size of the cecum might also be related to the amount of riboflavin synthesized. The following experiment was designed to determine whether or not such correlations could be made.

A total of 78 three-week-old male rats were fed isocalorically equivalent rations differing only in the kind of carbohydrate or in the amount of lard present. The following types of diets were employed (table 1): high sucrose (K-30), 40% fat, sucrose (K-31), high dextrin (K-32), 40% fat, dextrin (K-33), 40% lactose, sucrose (K-34), 40% fat, lactose (K-35), high-sucrose to which 10 parts of cellulose per 100 parts of ration had been added (K-36), 40% fat, sucrose, cellulose (K-37), high commercial cornstarch from which the dextrin had been prepared (K-38), and high alcohol-extracted dextrin (K-39). The last ration was fed to determine whether or not alcohol extraction would remove any hypothetical substance responsible for the stimulatory effect of dextrin. All animals received 6 μg. of riboflavin per day throughout the 6-week test period in order to insure consistent yet suboptimal growth. Half of the rats received the fullers' earth-treated butanol liver extract equivalent to 4% of the original liver powder, but because these animals responded no differently than did non-supplemented animals of the same group, no differentiation is made between the rats within each group. The rations containing high levels of fat were prepared daily and fed in clean food dishes to prevent rancidity.

At the end of 6 weeks the feeding of riboflavin was discontinued. The rats were maintained for an additional 9 days and throughout the last week of this period the feces were collected daily and stored in the refrigerator. The animals were then killed and the cecal contents collected quantitatively. The feces and cecal contents were dried in vacuo at 55°C., weighed, and saved for later riboflavin analysis.

The riboflavin content of the feces of individual rats was determined in duplicate using essentially a combination of the fluorometric methods of Hodson and Norris ('39) and Conner and Straub ('41) as outlined by Andrews ('43). Because of the small quantities of material available, the cecal contents of the animals of each group were combined for analysis. Several samples of feces were assayed by the bacteriological method of Snell and Strong ('39) as well as by the fluorometric method. The values obtained by the former procedure were erratic and as much as two or three times higher than those obtained by fluorometric analysis. This discrepancy was eliminated, however, by employing the Strong and Carpenter ('42) modification of the bacteriological assay which involves the removal of fatty stimulatory substances by means of ether extraction. The average riboflavin content of twenty samples of feces as measured by the fluorometric and bacteriological procedures was, respectively, 27.6 and 26.3 μg. per gram.

Lamoreux and Schumacher ('40) have reported that the riboflavin content of chicken excreta increases as much as 100% in 24 hours on standing at room temperature. That a similar synthesis of riboflavin did not occur in the rat feces during the collection intervals was shown indirectly by comparing the riboflavin content of the formed fecal material from the lower colon, obtained when the animals were autopsied, with that of the feces that had been collected throughout the preceding week. The colonic "feces" of eight animals taken at random from the various groups contained an average of 30.1 μg. per gram while the dropped feces from the same rats had a comparable riboflavin content of 32.9 μg. per gram. Active riboflavin synthesis probably does not occur in rat feces on standing because of their relatively low moisture content.

TABLE 4

Effect of various carbohydrates and of fat on growth and on the fecal excretion of riboflavin. All rats received 6 μg. of riboflavin per day except during period of feces collection.

RATION NO.	TYPE OF RATION	NO. OF RATS	GAIN IN 6 WKS.	RIBOFLAVIN/GM. OF FECES	RIBOFLAVIN EXCRETED IN FECES/WEEK	RIBOFLAVIN EXCRETED IN FECES/WK./100 GM. RAT	RIBOFLAVIN/GM. OF CECAL CONTENTS	CECAL CONTENTS/100 GM. OF RAT
			gm.	μg.	μg.	μg.	μg.	gm.
K-30	Sucrose	14	80	25.0	23.2	19.3	22.0	.15
K-31	Lard-sucrose	8	75	23.5	24.5	21.7	19.0	.16
K-32	Dextrin	8	108	34.3	72.4	50.3	25.6	.25
K-33	Lard-dextrin	8	68	24.3	24.3	22.8	19.5	.20
K-34	Lactose-sucrose	6	89	45.3	89.0	67.5	41.8	.48
K-35	Lard-lactose	6	87	23.4	50.6	38.8	25.6	.35
K-36	Cellulose-sucrose	6	83	6.7	28.3	23.1	9.7	.26
K-37	Lard-cellulose-sucrose	6	81	6.4	30.2	24.9	8.2	.24
K-38	Cornstarch	8	98	30.5	43.9	32.5	21.7	.14
K-39	Alcohol extracted dextrin	8	97	29.5	58.4	42.3	24.7	.31

It was obviously important to determine whether or not significant quantities of riboflavin were being contributed by the variable constituents of the diets. Lard, sucrose, cornstarch and lactose were found to contain less than 0.1 μg. of riboflavin per gram as measured by the fluorometric method, amounts that could not seriously affect the results of this experiment. The results are given in table 4.

The growth of rats receiving the high dextrin, cornstarch, and alcohol-extracted dextrin diets was superior to that of animals fed any of the other rations. Again the substitution of lard for a large part of the dextrin of the diet had a deleterious effect on growth while a similar

replacement of sucrose by lard was without effect. The inclusion of cellulose in the sucrose and lard-sucrose rations did not improve growth appreciably. The replacement of a large part of the sucrose of ration K-30 with lactose resulted in a slight increase in growth.

With the exception of the lactose-fed groups, growth is roughly proportional to the amount of riboflavin excreted per week in the feces. Similarly, but to a less marked degree, those groups of rats which exhibited the best growth, produced feces containing the greatest amount of riboflavin per gram. About the same amount of fecal riboflavin was excreted weekly by the animals receiving the high sucrose, fat-sucrose, fat-dextrin, and the cellulose diets. Although the riboflavin content of the feces of animals fed the cellulose diets was very low, the large amount of feces voided made the total excretion comparable to that of rats fed the high-sucrose diet without cellulose.

The feeding of the lactose-sucrose diet resulted in the greatest fecal production of riboflavin. According to the above correlation made between growth and the amount of riboflavin found in the feces, one might have expected the growth of the lactose-fed rats to have been correspondingly above that of the other animals, but this was not the case. It is possible, however, that when lactose was fed, the suboptimal riboflavin intake was not the primary growth-limiting factor. The data of Boutwell, Geyer, Elvehjem and Hart ('43) show that on a diet containing 48% lactose and an abundance of all the known vitamins, rats do not grow as well as when other carbohydrates are employed. When lard was substituted for the sucrose and part of the lactose of lactose-sucrose ration, K-34, the total fecal riboflavin was greatly reduced. Likewise the replacement of a substantial part of the dextrin of ration K-32 with lard resulted in a decreased fecal riboflavin output. The relatively low degree of intestinal synthesis of riboflavin which results when the high-sucrose (K-30) and sucrose-cellulose (K-36) rations are fed was not further decreased by the inclusion of high levels of fat in these diets.

With certain exceptions, the total amount of riboflavin excreted in the feces is related to the size of the cecum as measured by the dry weight of the cecal contents. The ceca of lactose-fed rats were greatly distended and contained whitish material, presumably lactose. The feeding of dextrin rations K-30 and K-39 also resulted in large ceca. It was in these animals that the greatest synthesis of riboflavin occurred. On the other hand, the feeding of cornstarch was responsible for abundant fecal production of riboflavin even though large ceca were not found. Conversely, the intestinal synthesis of riboflavin in

cellulose-fed rats was not great although the cecal size per 100 gm. of these animals was about the same as that of the rats receiving the high-dextrin ration. Apparently the amount of riboflavin excreted in the feces is as dependent upon the type as upon the quantity of material in the cecum. Although somewhat lower in most instances, the riboflavin values of the cecal contents are a reflection of those of the feces.

Effect of a high dietary intake of fat on the deficiency symptoms and survival of riboflavin deficient rats

It has been shown previously (Mannering et al., '41) that rats receiving a high fat-dextrin diet deficient in riboflavin develop a spastic paralysis of the hind quarters, a condition that does not occur with any marked severity when a high dextrin ration is fed. This same relationship of paralysis to fat intake can be shown when sucrose rather than dextrin is employed as the source of carbohydrate.

One group of rats weighing between 65 and 75 gm. received the basal high-sucrose ration K-30 and another, the lard-sucrose ration K-31. Animals of this size were employed in order to produce a chronic rather than the more acute state of deficiency which results when smaller rats are used, a higher incidence of spastic paralysis resulting when the deficiency is prolonged over many weeks. Animals were allowed to continue on experiment for 133 days throughout which time no riboflavin was administered. The high fat ration was prepared and fed daily to prevent rancidity.

Of six rats fed the high-sucrose diet three were still alive after 133 days. The average survival time was 118 days (83–133 days). Although several of these rats showed minor symptoms of spasticity before dying, in no instance could the condition be considered severe. None of the seven rats fed the lard-sucrose diet survived the 133-day period and the average survival time was 81 days (50–126 days). All of these rats developed severe paralysis.

These results were confirmed in a similar experiment. Eight of twelve rats fed the high carbohydrate diet survived the 133-day period, the average survival time being 113 days (85–133 days), as compared to 91 days (66–125 days) for twelve animals receiving the high fat diet. Again severe spasticity occurred only in rats fed the high lard ration. Three of the rats of each group were given 2 μg. of biotin per day (biotin liver concentrate) and three others received 10 mg. of inositol per day. Neither of these substances had any influence on survival or the deficiency symptoms. That the extremely emaciated condition of the

deficient rats was not responsible for the paralysis was shown by maintaining the weights of six rats receiving the high fat diet plus 100 μg. of riboflavin per day equal to the weights of six of the above rats fed the same ration without riboflavin by limiting the food intake of the former. Although emaciated, the starved animals receiving abundant riboflavin remained outwardly in excellent condition.

In addition to aggravating the production of spastic paralysis and decreasing the survival time the feeding of a high fat diet results in a more severe dermatitis and alopecia than is obtained when a high sucrose diet is fed. Denuded and incrusted areas are particularly noted about the face and limbs. Animals in the advanced stages of the deficiency have been cured by feeding 100 μg. of riboflavin per day. By the end of 2 or 3 weeks the spasticity completely disappears. Large clumps of a mixture of hair, skin and ration are pulled off by the rat leaving practically the entire surface of the animal bare. Gradually the hair grows in and the rat appears normal in all respects except that a few scars remain, especially about the face where incrustation is usually severe.

A condition known as "spectacle eye", characterized by a local alopecia immediately surrounding the eyelids of rats, has been related to biotin (Nielson and Elvehjem, '41) and to inositol (Pavcek and Baum, '41) deficiency. Chick, Macrae and Worden ('40) have noted a similar loss of hair in riboflavin deficient rats. A majority of the animals in our experiments developed this peculiar hair loss about the eyes which could not be prevented by the daily administration of 2 μg. of biotin or 10 mg. of inositol. These rats were commonly observed in the act of rubbing their eyes which probably accounts for the observed "spectacle eye".

The reddish accumulation of porphyrin on the paws, nose and whiskers of riboflavin deficient rats described by Chick et al. ('40) was observed in all but a small percentage of the above animals. The number of rats showing cataract was two out of eighteen fed the high carbohydrate diet and six out of nineteen receiving the high fat ration.

DISCUSSION

It is apparent from the foregoing experiments that dextrin and cornstarch reduce the dietary riboflavin requirement of the rat and that sucrose, cellulose, or fat do not share this property. Dextrin and cornstarch probably increase the amount of available riboflavin synthesized in the intestine by providing the intestinal microorganisms with a favorable medium for such synthesis, by increasing the number of

microorganisms, or by changing the flora to a type capable of producing greater quantities of the vitamin. The incomplete digestion of dextrin or cornstarch allows some of the carbohydrate to reach the lower regions of the tract where microorganisms are ordinarily found in abundance. Sucrose is more readily digested and absorbed than dextrin or cornstarch and would not be expected to reach this site of increased bacterial activity in any great quantity. This concept is in keeping with that suggested by Guerrant et al. ('35). Fat and cellulose, though capable of reaching the lower tract, are apparently of little value in promoting the synthesis of available riboflavin. In fact, fat may inhibit such synthesis.

The position of lactose in sparing riboflavin is uncertain, allowing growth intermediate between that obtained on the sucrose and dextrin diets. Morgan, Cook and Davison ('38) have reported that the feeding of lactose reduces the riboflavin requirement below that of animals fed either sucrose or cornstarch and were able to show that the ceca of lactose-fed rats contained appreciable amounts of riboflavin. Contrary to our observations, their data do not indicate that cornstarch supports better growth than does sucrose when the riboflavin intake is limiting.

Numerous investigators in the past have noted that the feeding of vitamin-low rations containing certain constituents results in greater growth than can be accounted for by the vitamin content of these constituents, and they have attempted to correlate such unusual growth with an increased vitamin content of the feces or of the material found in various sections of the digestive tract. Most of this work has not been of a strictly quantitative nature due largely to inadequacy of vitamin determination methods and to the inability to properly differentiate between the individual components of the B complex.

The idea has prevailed that a measurement of the vitamins in the tract and in the feces could somehow be used as a relative measure of the amount of the vitamin made available to the animal through bacterial synthesis. That such a relationship must necessarily exist is untenable on theoretical grounds. It can be successfully argued that the vitamin content of the feces or of other intestinal material may have little to do with the amount of the vitamin absorbed by the host. For example, the riboflavin may be synthesized in a region of the intestine from which it cannot be absorbed. Selye ('43) has studied the absorption of riboflavin from the various sections of the tract, separated by ligatures, using nephrectomized rats. He concludes that in the small intestine, riboflavin is both absorbed and excreted, while in the cecum and colon, injected riboflavin is rapidly destroyed with little,

if any, absorption of the vitamin. However, in his experiments very large concentrations of riboflavin were employed, and only amounts of the vitamin that could be detected grossly were considered. Whether or not minute amounts of riboflavin, such as are involved under normal conditions, can be absorbed from the cecum and colon still remains a question. That the cecum does actually contribute to the supply of the rat's B vitamins has been demonstrated in the cecectomy studies of Taylor, Pennington and Thacker ('42). The cecum has been emphasized as the most probable site of bacterial synthesis largely because of the abundance of microorganisms found there. However, other sections of the tract cannot be ignored in this respect. Porter and Rettger ('40) find, contrary to much prevailing opinion, that the upper intestine of the rat contains appreciable numbers of viable bacteria.

Secondly, the vitamin may exist as an integral part of the bacteria, unavailable to the animal. Abdel-Salaam and Leong ('38) have demonstrated that the thiamine synthesized by the mixed flora taken from the ceca of rats and grown in vitro is contained in the bacterial cells rather than in the surrounding medium, and Mitchell and Isbell ('42) find that the greater proportion of the vitamins found in the cecal contents of rats is contained in the bacterial cells. However, it has been shown that when bacteria, capable of synthesizing vitamins, including those species normally found in the tract, are grown in pure cultures, the vitamins synthesized are found largely in the medium (Burkholder and McVeigh, '42, Thompson, '42, Rodgers, '42). The vitamins elaborated are a result of both bacterial excretion and autolysis. This difference between the amount of free vitamin obtainable when mixed and when pure cultures were used is understandable if, in the mixed cultures, bacteria requiring or destroying an external source of the vitamin are present as well as those capable of its synthesis, in which case, the former would utilize the vitamin released by the latter. Comparable symbiotic relationships may be expected to exist in the cecum and other parts of the tract containing a mixed flora.

The contents of the tract may be considered to be in constant state of activity, bacteria constantly dividing, dying, disintegrating, and giving up their contents for digestion and absorption. Organisms may be excreting vitamins directly into the intestinal media to be absorbed with varying degrees of rapidity by either the host or by still other bacteria requiring the vitamin. The state of the intestinal contents at any given instant, then, will be the result of the relative rates at which the many possible processes proceed. A small quantity of either total or available vitamin in the intestinal material or feces might be inter-

preted to mean that large amounts of the vitamin had been formed and absorbed as well as that a small quantity had been produced.

In view of the above considerations, it is understandable that in our experiments, growth could not be unconditionally correlated with the fecal riboflavin excretion. Perhaps it is more surprising than otherwise that the correlation was as good as it was, the only exception being that of the groups receiving lactose.

It is important that investigators conducting vitamin balance studies recognize that riboflavin is produced in significant quantities by bacteria in the tract and that the amount of riboflavin in the feces is largely independent of the amount fed in the diet. Sure and Ford ('42) overlooked this fact and considered the fecal excretion of riboflavin to be unabsorbed vitamin of dietary origin. Vitamin balance studies such as those of Mitchell and Isbell ('42) do not give accurate values for the amounts of the B complex obtained by the rat from an enteric source because the degree of destruction of these vitamins in the tissues is not known.

Though little difference can be shown between the growth of riboflavin deficient rats receiving sucrose and high fat-sucrose diets, fat aggravates the production of spastic paralysis, suggesting a role of riboflavin in nerve metabolism. Nielson and Elvehjem ('42) have reported a similar spasticity in biotin deficient rats receiving abundant riboflavin. The two conditions are not identical, however, in that biotin failed to prevent the paralysis resulting from the lack of riboflavin.

While studying the sparing action of fat on thiamine, Evans, Lepkovsky and Murphy ('34) observed that rats continued to grow on a 55% fat ration deficient in thiamine but adequate in vitamin G, and that if vitamin G was discontinued, the animals developed what were described as typical symptoms of beriberi. They concluded that vitamin G prevented these symptoms, and in effect, actually spared thiamine. It now seems more likely that these workers had observed the paralysis due to riboflavin deficiency rather than the symptoms of thiamine avitaminosis.

SUMMARY

When the riboflavin intake is sub-optimal, rats receiving a diet rich in dextrin or cornstarch show greater weight increases than do animals receiving diets characterized by their content of sucrose, cellulose, lactose, or lard. Lactose is not entirely without effect in this respect.

The total fecal output of riboflavin of rats receiving various riboflavin-low diets has been determined, and in general, those carbo-

hydrates which decrease the amount of dietary riboflavin needed by the rat for growth are also responsible for the greatest quantities of riboflavin in the feces. However, the fecal excretion of riboflavin was greatest when lactose was fed, although the growth-promoting effect of lactose was not great.

The relationship between growth, cecal size, intestinal synthesis, and the quantity of riboflavin in the cecum and feces is discussed.

The feeding of high levels of dietary fat to riboflavin deficient rats results in a spastic paralysis of the hind quarters, a condition not noted with any degree of severity when riboflavin-low rations of high carbohydrate content are employed. Riboflavin deficient rats survive for shorter periods of time when fed a high fat ration than when maintained on a high carbohydrate diet.

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FOOD UTILIZATION AND APPETITE IN RIBOFLAVIN DEFICIENCY [1]

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THREE FIGURES

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Several papers have appeared recently dealing with riboflavin as a factor affecting the efficiency of food utilization in the rat. Sure and Dichek ('41) and Sure ('41), employing a paired-feeding technique, have shown that riboflavin has a pronounced effect on the utilization of food for the synthesis of body tissues. It was further demonstrated that, unlike in a thiamine deficiency, the final collapse of the riboflavin deficient rat is not associated with any great decrease in food intake. Voris, Black, Swift and French ('42), using moderately deficient animals, have made similar observations.

In the paired-feeding studies of the above-mentioned authors, animals receiving an adequate diet were limited in their food intakes to the amounts eaten by animals fed the deficient diet. Here the efficiency of food utilization was measured by a comparison of the growth of the two groups of animals. It is also possible to obtain information regarding food utilization by means of ad libitum feeding if groups of animals are given graded, suboptimal levels of the vitamin in question and the amount of growth per calorie of food consumed computed for each group. During the course of the experiments described in the preceding paper (Mannering, Orsini and Elvehjem, '44) we were able to study the effect of riboflavin on food utilization using this latter procedure.

The composition of the riboflavin-low rations has already been given (Mannering et al., '44). Three-week-old male albino rats weighing 35 to 40 gm. were partially depleted of their riboflavin reserves for 2

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weeks on ration K-24 before being divided into four groups of twelve animals receiving rations K-24, K-25, K-26, and K-27 and varying amounts of riboflavin (0, 3, 6, 9, and 12 μg. per day). These rations differ only in the percentage or type of fat present and are calorically equivalent. Food consumption records were kept throughout the 7-week supplementation period. In figure 1 both the average grams gained per day and the average grams gained per 100 Cal. fed have been plotted against the number of micrograms of riboflavin fed per day. To conserve space the data for all rats receiving a given level of riboflavin were averaged together regardless of the type of ration fed. This was justifiable since equal numbers of rats were employed at the various riboflavin levels for each of the rations. The curves shown are qualitatively similar to those obtained when the four groups are considered separately.

It is seen that as the daily administration of riboflavin is increased, less food is necessary for a given increment of growth. The curve representing growth is linear within the limits of 0 and 12 μg. of riboflavin per day and would be expected to level off if greater amounts of the vitamin were fed. The configurations of the curves indicate that food utilization approaches a maximum at a lower level of riboflavin intake than does growth. When the average grams gained per 100 Cal. fed was plotted against the logarithm of the number of micrograms of riboflavin fed per day, a straight line was obtained (fig. 1).

The marked effect of riboflavin in supporting the utilization of food for growth cannot be accounted for by a lack of absorption. The feces of all rats receiving rations K-24 and K-27 were collected during the third and fourth weeks following the depletion period, dried, and weighed. The total digestibility of these rations was excellent. The average grams of feces obtained per 100 gm. of ration K-24 fed was 3.1 (1.2–4.8) and of ration K-27, 5.2 (4.0–6.4). Variations bore no relation to the riboflavin intake.

A second experiment was devised for the purpose of studying the effect of riboflavin on the efficiency of food utilization for maintenance. A method of "paired-weighing" was employed whereby an animal receiving a complete diet was restricted in food intake so that its weight remained equal to that of an ad libitum-fed rat receiving a diet deficient in riboflavin. A comparison of the efficiency of food utilization was then based on the amount of food necessary to maintain each of the animals at the same weight. So little growth occurs in a rat completely deprived of riboflavin that food is used almost entirely for maintenance.

Six male albino rats weighing between 65 and 75 gm. were fed 100 μg. of riboflavin per day and just enough of ration K-31 to keep their

weights equal to the weights of six similar rats fed the same ration ad libitum but receiving no riboflavin. By means of daily weighings and careful allocation of food it was possible to keep pairs of starved and deficient rats within 3 gm. of each other. During the eleventh week three of the deficient animals died while the remaining three collapsed during the fifteenth, sixteenth and seventeenth weeks. All of the starved animals remained in apparent good condition throughout the experiment

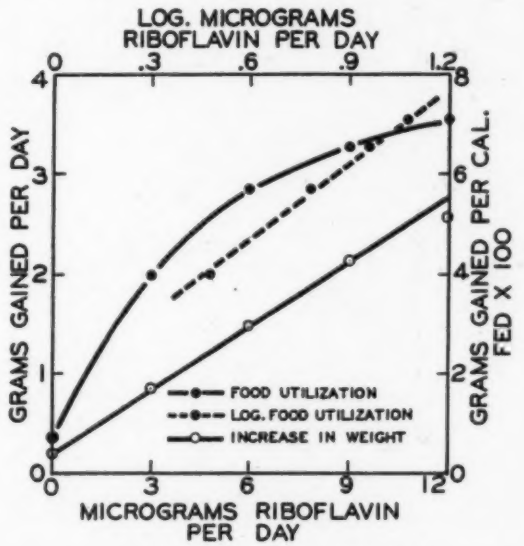

Fig. 1 Curves showing effect of progressively higher levels of riboflavin on growth and food utilization of rats during a 7-week period following 2 weeks of depletion on a riboflavin-low diet. Each point represents twelve rats.

despite extreme emaciation. In figure 2 are shown the growth curves and weekly food intakes of both deficient and starved rats for the first 10 weeks of the experiment.

After the first week the starved animals required progressively less food to maintain their weights equal to those of the riboflavin deficient rats until by the tenth week the latter were consuming an average of 1.8 (1.2–2.8) times more of the ration than the former. During the entire experimental period of 17 weeks the avitaminotic rats ingested an average of 1.5 (1.3–1.7) times as much food as the starved controls. The food utilization during the fifth through the eighth weeks is more strictly representative of food utilization for maintenance than during any other period of the experiment because the animals were neither

gaining nor losing weight at this time. Throughout this growth plateau period the rats that were deprived of riboflavin consumed an average of 1.6 (1.3–1.9) times the amount of food eaten by their control mates.

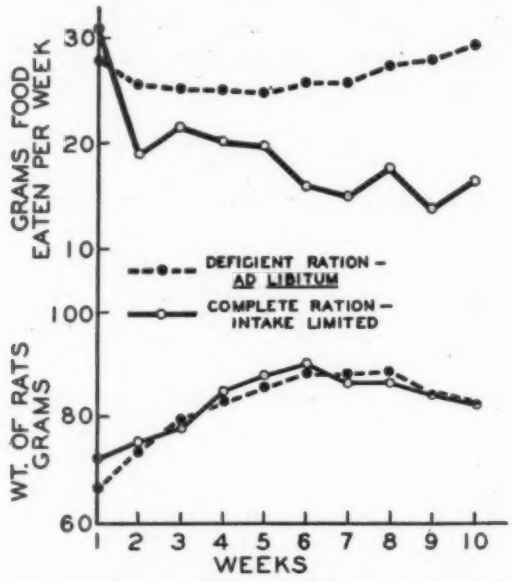

Fig. 2 Curves showing growth and food consumption of riboflavin deficient rats and of rats receiving a limited intake of an adequate diet. Each curve represents six rats.

Figure 3 shows the weekly food consumption per 100 gm. of rat of both starved and deficient animals. During the first 4 weeks of the experiment the rats deprived of riboflavin consumed progressively less food per 100 gm. of weight and then progressively more as the deficiency became more severe. The period of increased efficiency of food utilization occurred while the animals were slowly growing, while the later decrease in efficiency corresponded to the period when the rats were losing weight. The starved animals became continuously more efficient in their utilization of food as the period of restricted food intake was prolonged.

Rats that are limited in food intake but supplied with sufficient riboflavin were observed to be much more active than deficient rats. Unless the energy output of the animal is known, it is, of course, impossible to obtain absolute values for the influence of riboflavin on food utilization for maintenance. Because the starved animals were apparently more active than the vitamin deficient rats, the effect of

riboflavin in promoting the utilization of food is probably even greater than has been indicated.

It has been previously mentioned that certain workers have been unable to find any appreciable inanition in riboflavin deficiency. The following study indicates that moderate inanition does occur in most animals suffering from a lack of riboflavin.

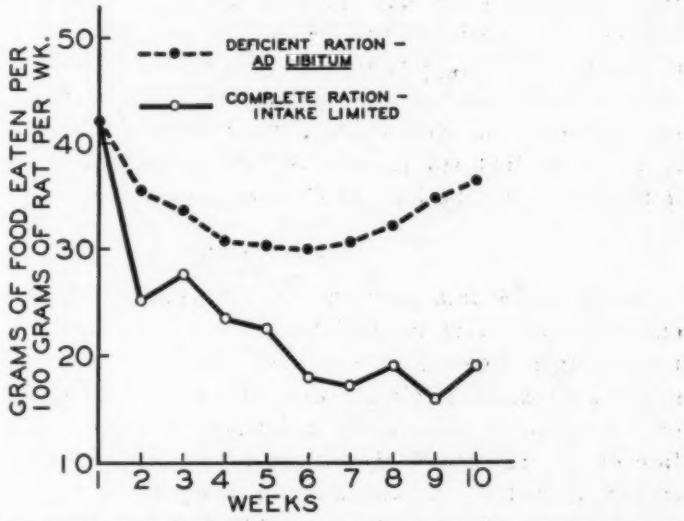

Fig. 3 Curves showing the grams of food consumed per 100 gm. of rat per week of starved and of riboflavin deficient rats. Each curve represents six rats.

Male albino rats weighing about 65 gm. were divided into two groups, one of which received ration K-30, and the other, ration K-31. No riboflavin was fed during the 19-week experimental period. The series was repeated, but as a separate consideration of either the two series or of the animals receiving the different rations did not add to the information gained, the results have been combined. A total of thirty-seven rats were employed in this experiment. By the end of the fifth week of depletion the rats were consuming an average of 20% less calories than they were during the first week of the experiment. On a calculated basis of Cal. eaten per 100 gm. of rat, an average decrease of 35% was observed during the fifth week. The selection of the fifth week for purposes of comparison was arbitrary and does not necessarily represent the period of lowest food intake. During the nineteenth week, eleven surviving animals were consuming an average of 27% less calories than they were during the first week, an average decrease of 31% per

100 gm. of rat. Considerable variation was noted in the degree of anorexia, some rats even showing an increased appetite. An example of this variation is evident in figure 2, where, by coincidence, the six rats used in the experiment represented by the graph did not show appreciable inanition.

It was often observed that rats markedly increased their food intake for a period of a few days prior to dying of the deficiency. This has also been reported by Sure ('41). The increase in food consumption during the last 7 days before death over that of the preceding 7 days was calculated and found to average 11%. The average, however, does not well represent those animals which showed increases just previous to dying in that several of the rats actually decreased their food intake. Increases as high as 62% were observed.

DISCUSSION

The presented data clearly demonstrate that riboflavin has a profound effect on food utilization both for growth and for maintenance. It has been further shown that a lack of riboflavin is not associated with severe anorexia, the animals consuming considerable quantities of food even in the later stages of the deficiency. The question arises as to the fate of the ingested food, a large proportion of which, although absorbed, cannot be accounted for as being utilized for growth or for maintenance. The most logical explanation for the poor food economy in riboflavin deficiency is that the intermediate products of metabolism are probably wasted through incomplete combustion. The role of riboflavin in the function of enzyme systems controlling cellular respiration has been well established (Elvehjem and Wilson, '40; Potter, '40; and Green, '41). This view also gains support from the observation of Orsini, Waisman, and Elvehjem ('42) that the respiratory quotient of the riboflavin deficient rat is abnormally high.

SUMMARY

Riboflavin plays an important role in the economy of food utilization both for growth and for maintenance.

Moderate inanition occurs in riboflavin deficiency but is not responsible for the death of the deficient rat since a relatively high level of food consumption is maintained throughout the entire period of avitaminosis. The food intake often increases during the last few days preceding death.

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ADEQUACY OF SIMPLIFIED DIETS FOR THE PIG

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ONE FIGURE

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The literature on the nutritional requirements of swine is too voluminous for a detailed review but it has been shown that these animals require vitamins A (Hughes, Aubel, and Lienhardt, '28) and D (Johnson and Palmer, '41), thiamine (Wintrobe, Mitchell, and Kolb, '38), riboflavin (Hughes, '40), pyridoxine (Hughes and Squibb, '42), pantothenic acid (Hughes and Ittner, '42), and nicotinic acid (Chick, Macrae, Martin and Martin, '38 a). Hughes and collaborators ('42) reported normal growth in swine that did not receive choline and according to Wintrobe and coworkers ('42) it is uncertain that swine require this substance.

The first use of simplified diets for studies on the nutritional requirements of swine, was published by Chick, Macrae, Martin, and Martin ('38 b). Vitamins A and D were supplied in cod liver oil. Thiamine, riboflavin, and nicotinic acid were supplied as crystalline compounds. The pigs were 12 weeks of age when the experimental period began and they weighed from 51 to 70 lb. Gains in weight were normal for a short time but ceased abruptly after 3 or 4 weeks. If 4% of dried yeast was incorporated in the diet growth was continuous.

Wintrobe ('39) also was successful in rearing pigs on synthetic diets, though the water-soluble vitamins were supplied in dried yeast. It is noteworthy that he started the pigs at an earlier age than did the investigators previously mentioned. The pigs were from 2 to 23 days of age in the beginning, and they were retained until they were approximately 8 months old. During the first 3 months they gained in weight very slowly, but following that time they began to grow normally. Wintrobe, Miller, Follis, Stein, Mushatt, and Humphreys ('42) repeated this observation, with minor changes in procedure. The yeast feeding was discontinued when the pigs were approximately 12 to 15 weeks of age, and synthetic vitamins were included in the diet. These

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series no. 955.

included thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, and choline. The pigs grew fairly well for about 6 months, though far under the normal rate. One would conclude that the diet was not entirely adequate.

Hughes and coworkers ('42) used a basal diet composed of casein 15, sucrose 81, and salts 4, but their animals grew normally although they received no crude vitamin carriers. Vitamins A and D were supplied in cod liver oil. The water soluble vitamins included were thiamine, riboflavin, pyridoxine, pantothenic acid, and nicotinic acid. It should be noted, however, that the animals weighed about 30 lb. when the feeding test began and presumably this was shortly after they had been weaned.

EXPERIMENTAL

The mortality had been excessively high during preliminary trials, in which the new-born pigs were removed from the sows at birth, and it was assumed that the failure to receive colostrum was at least partly responsible for the deaths. The pigs were therefore allowed to

TABLE 1

Illustrative basal ration — ration 308 — used for feeding experiments with pigs.

INGREDIENT		VITAMIN[1] SUPPLEMENTS	
	%		*mg./100 gm. diet*
Acid washed casein	30	Thiamine	0.8
Sucrose	30	Riboflavin	1.6
Corn starch	5	Pyridoxine	1.2
Lard	30	Ca-pantothenate	2.0
Salt mixture[2]	5	Percomorph oil	50.0 (3000 I. U. vitamin A, 850 I. U. vitamin D)
		Nicotinic acid	2–4
		Alpha tocopherol	4
		K_1	2
		Choline	200–300

[1] Generously supplied by Merck and Co., Rahway, New Jersey.

[2] 99.2% of the salt mixture 351 of Hubbell, Wakeman and Mendal ('37), 0.75% manganous sulfate, 0.05% of zinc sulfate.

nurse their dams for the first 2 days after birth and were then given the experimental diets. These were all of the simplified type. The diet supplied to most of the pigs contained the four fat-soluble vitamins and six members of the vitamin B complex. A typical example is described in table 1.

Diet 308 was modified at various times but apparently one diet was as useful as another. The salt mixture of Hubbell, Wakeman, and

Mendel is low in phosphorus, but the addition of more phosphorus did not improve the diet. Some attention to detail is necessary to convert the diet into a liquid that is suitable for very young animals. The casein was added to warm water, with enough sodium hydroxide (0.75 gm. of the alkali per 30 gm. casein) to dissolve it. The lard was melted and incorporated into the casein solution with a mechanical stirrer. The starch was dissolved in boiling water and it, along with the salt mixture, was added to the protein-fat emulsion. The sucrose and vitamins were added last, with enough water to lower the dry matter content to 19%. The entire mixture was then strained, and run through a homogenizer under a pressure of 2500 lb. The pigs were hand-fed, eight times daily, from a bottle equipped with a rubber nipple. The diet was warmed to about 40°C. at each feeding. The pigs were quartered in small pens, on wooden floors, with shavings for bedding. It is essential to keep young pigs warm; therefore the temperature of the room was kept at approximately 75°F. A few pigs were retained past the age when they would normally be weaned, and when they weighed 25–30 lb. they were given their rations in dry form. Undoubtedly the transfer could be effected much sooner. For convenience the results will be presented in two sections.

Adequacy of diets which contain no water-soluble vitamins except the synthetic compounds

The mortality rate was rather high during the first few days the pigs received the experimental diets. In all probability many of the deaths were due to the fact that the diet is partially inadequate, but in most cases the early deaths were precipitated by failure of the pigs to consume the diet. Animals will not consume normal amounts of a deficient diet after the deficiency becomes effective. When it first became apparent that diet 308 is inadequate the four water-soluble vitamins it does not contain were added, singly or in combination. The more significant data are summarized in table 2. The essential details are brought out more clearly in figure 1.

The animals shown in table 2 are grouped according to the number of vitamins in the diet, and according to the dates when they were under observation. However, a considerable number of the animals in group IIa were started without adding one at the same time to group IIb. There were thirty-four animals in all and fifteen of them died, though a few of the deaths occurred after the close of the experimental period. Most of the others made slow gains in weight until they were 4 or 5 weeks old.

Of the pigs in group Ia, all under observation at the same time, one made practically no gain in weight and died. Another made moderate gains in weight and was normal in appearance, but was dropped from the experiment after 25 days to conserve food. The third pig gained at somewhat less than the normal rate for 18 weeks when the experimental feeding was discontinued. The hair and skin were noticeably coarse and rough but there were no other signs of malnutrition and no reason to suppose the pig would not attain a more or less normal maturity. Despite the good record made by this pig it is certain that rations such as no. 308 are partially inadequate during the first few days or weeks of a pig's life. The higher degree of success attained

TABLE 2

Diets which contain no water-soluble vitamins except synthetic compounds are inadequate.

GROUP		ADDITIONS TO RATION 308	NO. OF PIGS	EXPERIMENTAL PERIOD	INITIAL WEIGHT	AVERAGE DAILY GAIN	
				days	*lb.*	*lb.*	
			1	128	3.7	0.56	
	a	none	1	25	3.1	0.30	normal
			1	19	3.0	0.04	
I							
	b	p-amino-benzoic acid	1	26	3.0	0.35	
			4	12–26	3.0	0.19	
	a	none	1	89	4.0	0.18	
			20	10–58	3.0	0.07	
II							
	b	ascorbic acid, biotin, inositol, p-aminobenzoic acid	5	3–11	2.7	0.09	

by Hughes ('40) is probably due to the fact that he began with pigs which were much older and which had intestinal flora of a different type, and the intestinal bacteria were able to synthesize the unrecognized vitamins required by swine.

A preliminary trial had given some indication that p-aminobenzoic acid would improve ration 308, and for that reason it was investigated more intensively. Subsequent studies indicated that the ration was not improved by including this compound (Ib). Later on the other available vitamins, or substances reported to be vitamins, were included. The amounts added in mg. per 100 gm. of diet were, respectively, ascorbic acid 30, inositol 30, p-aminobenzoic acid 30, and biotin 0.03.

Five pigs received ration 308 plus these four substances, but there was no evidence that the added vitamins improved the diet in any respect. Other pigs, which will not be described, received one, two, or three of these vitamins, in addition to ration 308, but as would be expected the results were no better than when all four were supplied together. It was concluded that the pig either does not require any of these four vitamins, or else an unrecognized vitamin was the first limiting factor.

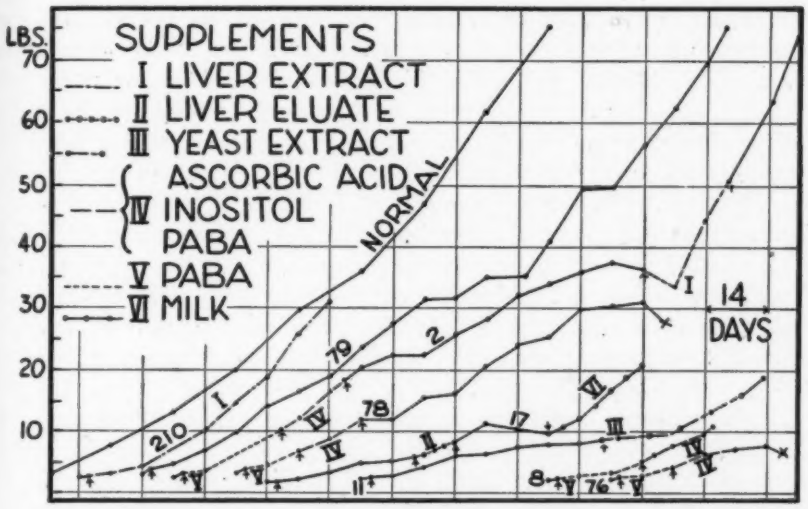

Fig. 1 As a rule pigs grow slowly or fail on ration 308, indicated by an unbroken line. Pig 79 is an exception. Pigs grow normally if diet 308 is fortified with liver or yeast extracts (pigs 2, 210, and 11). A short trial indicated that the active agent of liver extract can be adsorbed on fuller's earth and eluted (pig 17). Ration 308 is not improved by the addition of ascorbic acid, inositol, or p-aminobenzoic acid (pigs 2, 76, 78).

As was mentioned before it has been shown that vitamins A and D, and thiamine, riboflavin, pyridoxine, pantothenic acid, and nicotinic acid are required by swine. Decisive data on the requirement of this animal for choline and vitamins E and K are not available, and an attempt was made to determine whether these vitamins are required by the pig. The experimental diet was the same as ration 308 except it did not contain these three vitamins. Since the results were negative the data will not be reported in detail. It is sufficient to say that the pigs made very slight gains in weight but they gained as rapidly as did the controls, and there were no specific symptoms of any deficiency.

The data do not indicate that the pig requires any of these three vitamins. This interpretation is well supported since satisfactory rations which contain no vitamin E, vitamin K, or choline were described by Hughes and Ittner ('42). Wintrobe and coworkers ('42) reared normal pigs on rations which presumably contained neither vitamin E nor vitamin K.

The characteristic symptoms of pigs fed the basal diet, ration 308, are as follows. All pigs developed rather severe diarrhoea within 24 hours after they were placed on experiment, and some of them never recovered from this disorder. However, after about a week, most of the surviving animals did recover and a few grew fairly well for as long as 2 to 5 weeks. Following this time their feed consumption was irregular, the diarrhoea returned, they became rough and unthrifty, and the rate of growth declined. Some of the pigs developed a sticky, dirty exudate around the eyes and mouth and on the inside of their legs. If the diet was not changed the symptoms became more and more severe, and the pigs eventually died. Post mortem examination failed to disclose any characteristic abnormalities. The liver and kidneys of all the pigs fed ration 308 appeared to be normal. Pneumonia was the immediate cause of death in a few of the pigs that died within the first 3 or 4 weeks after they were placed on experiment. Peritonitis was the immediate cause of death of some of the pigs which survived for 1 or 2 months. Necrotic and inflamed areas were found in the intestinal tract of a few animals.

Adequacy of diets which contain crude vitamin carriers as sources of the unrecognized vitamins required by swine

After some of the pigs on ration 308, or one of its modifications, had begun to decline and it seemed certain that these animals require a factor or factors in addition to the vitamins now recognized, a variety of crude vitamin carriers was added to the diet. This was done to show that the failures were not due to disease and that the pigs would grow if the ration was adequate. Furthermore it would be helpful in future work to have data concerning crude vitamin carriers that are good sources of the missing factor or factors. The effectiveness of these crude preparations was usually tested by the curative method, but in a few cases they were supplied from the beginning. Extracts of yeast and liver were used most extensively, and they are described below.

A. An aqueous extract of brewer's yeast concentrated to contain 50% dry matter and centrifuged to remove suspended solids.

B. An aqueous extract of beef liver concentrated to a low volume and treated with an equal volume of 95% ethanol. The filtrate was concentrated to a syrup (O'Dell and Hogan, '43).

C. An aqueous extract of fresh pork liver concentrated to yield a dry matter content of 30–50%.

D. An alcoholic extract of beef liver, similar to fraction 4303 of Hogan, Richardson, Patrick, and Kempster ('41).

E. An aqueous extract of beef liver, similar to fraction 4080 of Hogan, Richardson, Patrick, and Kempster ('41).

F. An aqueous extract of beef liver adsorbed with fuller's earth at pH 1, and the adsorbate eluted with 0.2 N ammonium hydroxide. This preparation is the fuller's earth eluate of Richardson, Hogan, and Karrasch ('42).

Whenever an extract was included in a ration it replaced sucrose. All replacements and calculations are based on dry weight. The data obtained with these extracts are shown in table 3. Each record is for an individual animal.

It will be observed in table 3 that the yeast extract, A, was moderately effective in increasing the growth rate of pigs which had developed symptoms of malnutrition while consuming ration 308 or some ineffective modification. The response of the pigs which received liver extracts gave the impression that these preparations were more potent than was the yeast extract. However, when pigs consumed either one the diarrhoea disappeared, the food intake and rate of growth increased, and there was marked improvement in appearance. The activity of fraction F, the eluate of a fuller's earth adsorbate of liver extract, is worthy of special mention. Since the supply was limited it was supplied for 9 days only, but after it was included in the diet the rate of gain immediately increased. The change in the appearance of the pig was far more striking than the change in weight. It was a sick animal before the change was made but 9 days later the pig was apparently normal. Two weeks after the feeding of the eluate was discontinued, however, it had a poor appetite, had developed severe diarrhoea, and was becoming emaciated. It recovered again, and began to grow normally on being transferred to a diet of milk. Three pigs received the combination of extracts D and E from the time they were 2 days old. They grew as well, and were as normal in appearance as were the control pigs which received fortified cow's milk. The results with liver extracts provide additional evidence that young pigs require an unidentified nutritional factor. Some additional details,

and a normal growth curve (Ittner and Hughes, 38), are shown in figure 1.

It seems worthy of mention that cow's milk, at least some samples, is slightly deficient for the pig during the first few weeks of life. The symptoms noted were weakness in the hind legs, an exudate around the eyes, a rash on the flanks, and a rough hair coat. However, the pigs regained a normal appearance by the time they were 8 weeks old, and were as heavy as their litter mates that had been nursed by their mothers.

TABLE 3

Extracts of yeast and liver as sources of unrecognized vitamins required by the pig.

EXTRACT Type	AMOUNT IN DIET	EXPERIMENTAL PERIOD	INITIAL		DAILY GAIN BEFORE AND AFTER EXTRACT WAS SUPPLIED	
			Age	Weight	Before	After
	%	*days*	*days*	*lb.*	*lb.*	*lb.*
A: brewers' yeast, aqueous	5	20	31	4.9	0.06	0.32
	5	36	54	8.7	0.12	0.26
B: beef liver, aqueous treated with 95% ethanol; filtered; filtrate used	5	21	106	37.9	0.15	0.62
C: pork liver, aqueous; conc. to give 30–50% dry matter	5	20	61	10.3	0.11	0.32
	5	20	40	7.7	0.12	0.44
D: beef liver, alcoholic extract similar to fraction 4303 (Hogan et al., '41)	5	35	21	4.3	0.06	0.24
D:	2.5	54	2	3.2	...	0.53
E: beef liver, aqueous extract similar to fraction 4080 (Hogan et al., '41)		30	2	2.7	...	0.31
	5	30	2	2.5	...	0.32
F: beef liver, aqueous, fuller's earth adsorbate eluted with 0.2 N ammonium hydroxide; same as fuller's earth eluate of Richardson et al., '42	1	9	33	6.0	0.15	0.30
Entire diet fortified[1] cow's milk		51	5	2.3	...	0.56
		51	5	2.5	...	0.47

[1] One liter whole milk + 50 gm. whole milk powder, + 50 mg. iron + 4 mg. copper + 5 mg. manganese + 1.5 mg. iodine.

DISCUSSION

The nutritional requirements of swine are of great practical importance, but our chief interest at present is in comparing these requirements with those of other species. In this respect animals may for the present be divided into two groups. (1) The mouse, rat, and hamster grow normally on diets which contain no water-soluble vit-

amins except those now recognized. (2) The guinea pig, rabbit, chick, and pig do not grow normally unless their diets include one or more unrecognized vitamins. In the absence of precise information, it is safer to assume, when estimating the adequacy of a diet, that man belongs in this second group. There are excellent reasons for believing that these animals do not all require the same unrecognized vitamins, but it is of practical interest that some of the more effective vitamin carriers contain all unrecognized vitamins of which we have any knowledge. For example they are all present in a water extract of fresh liver.

SUMMARY

1. Pigs seldom grow normally, and few survive, if they are transferred at 2 days of age to simplified diets that contain no water-soluble vitamins except those now recognized. Occasionally, however, a pig will grow fairly well on such a diet.

2. No evidence was obtained that the pig requires vitamin E, vitamin K, choline, biotin, inositol, or p-aminobenzoic acid, but it may be impossible to reach a final decision in this matter until all essential unrecognized vitamins are available and have been tested.

3. Water extracts of liver or yeast contain all unrecognized vitamins required by the pig.

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STUDIES OF UNIDENTIFIED VITAMINS REQUIRED BY THE CHICK

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Bauernfeind, Schumacher, Hodson, Norris and Heuser ('38) presented evidence of a new chick growth factor which they found not to be identical with any of the known vitamins. Schumacher, Heuser and Norris ('40) showed that the new growth factor of Bauernfeind and associates ('38) was a complex consisting of at least two components which they designated factor R and factor S. Record and Bethke ('42) presented evidence indicating that one of the factors of Schumacher and associates ('40) might be choline.

In further studies on factors R and S of Schumacher, Heuser and Norris ('40), evidence has been obtained which confirms the existenee of these factors and shows that neither one of them is identical with choline, folic acid or vitamin B_c. If folic acid, the Lactobacillus casei factor, and vitamin B_c are the same substance, as the recent results of Pfiffner and associates ('43), Stockstad ('43) and Mitchell and Williams ('44) indicate, a second chick antianemic factor, distinct from factors R and S, has been revealed. The results of these studies are presented in this report.

EXPERIMENTAL

Preparation of factor R and S concentrates

Strain S dried brewers' yeast[2] was used in the preparation of concentrates of factors R and S. Three methods of extracting yeast were employed. (1) Water extracts were prepared by heating a uniform suspension of 5 kilograms of yeast in 25 liters of water at 80–85°C. for 1 hour with constant stirring. The insoluble residue was removed by filtration and reextracted under the same conditions. (2) Acid extracts of yeast were prepared by the same procedure using 0.12 N HCl. The acid extraction mixture was adjusted to pH 6.5 with 40% NaOH before each filtration. (3) Digestion extracts were prepared by treatment of

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[2] Anheuser-Busch, St. Louis, Missouri.

yeast with takadiastase.[3] One kilogram of yeast was suspended in 10 liters of water, 10 gm. of takadiastase were added and the pH was adjusted to 4.5 with acetic acid. The mixture was incubated for 24 hours at 37°C. under benzene, brought to pH 6.5 with NaOH, steamed for 30 minutes, cooled and filtered. The insoluble residue was washed by resuspension in 10 liters of water and filtered.

The combined filtrates from each extraction of yeast were concentrated under reduced pressure (40–50 mm. Hg). The syrup was brought to pH 1.6 by addition of concentrated HCl and the volume was adjusted so that 1 ml. was equivalent to 2 gm. of original yeast. To the acidified concentrate 10 volumes of 95% ethanol were added slowly with constant stirring. Stirring was continued for 30 minutes and the mixture was allowed to stand overnight. The precipitate containing factor S (fraction 1), which formed in the acid-alcohol mixture, was removed by filtration. The acid-alcohol filtrate was brought to pH 7.0 by addition of 40% NaOH with constant stirring. The neutral alcohol mixture was allowed to stand overnight and the precipitate containing factor R (fraction 2) which formed was removed by filtration. The neutral alcohol filtrate (fraction 3) containing folic acid but free of factor R and factor S as determined by growth studies, was concentrated under reduced pressure to a small volume in preparation for feeding.

Experiments with simplified diet 543

A modification of diet 540 of Schumacher, Heuser and Norris ('40) was used in these studies. The composition of this diet, designated simplified diet 543, is given in table 1. The average choline content of this diet was found to be 0.08%. With the added choline it contained 0.18% choline. The extra choline was made necessary by the discovery of Record and Bethke ('42) and by observations made at this laboratory that diet 540 was deficient in this factor. The results of experiments conducted at this laboratory[4] showed that the addition of 0.2% choline to diet 543 was no more effective than 0.1% in promoting growth and preventing perosis in chicks. Hegsted, Mills, Elvehjem and Hart ('41) have reported that the choline requirement of the chick is approximately 0.1%.

Three experiments were conducted to determine the growth activity of the fractions prepared from the different yeast extracts. Fractions 1, 2 and 3 were each fed at levels equivalent to 5, 10 and 15% of yeast,

[3] Parke, Davis and Company, Detroit, Michigan.

[4] Unpublished results.

and in all possible combinations at levels equivalent to 10% of yeast. All lots of each experiment contained fifteen White Leghorn chicks of mixed sex. The summarized results are presented in table 2. The mortality in these experiments was not excessive and was somewhat lower with yeast and fraction 2.

TABLE 1

Composition of basal diets.

INGREDIENT	DIET 543	DIET 653
	gm.	*gm.*
Degerminated yellow corn meal	66.65	...
Corn starch	....	51.7
Purified casein	10.00	27.5
Peanut oil meal	15.00	...
Gelatin	....	7.5
Salt mixture	5.00 [1]	5.0 [2]
Soybean oil	3.00	4.5 [3]
Cellophane	....	3.0
Fish liver oil	0.25 [4]	0.5 [4]
Choline chloride	0.10	0.2
Inositol	....	0.1
	mg.	*mg.*
p-Aminobenzoic acid	....	10.0
Nicotinic acid	....	3.0
Calcium d-pantothenate	0.7	1.63
2-Methyl-1, 4-naphthoquinone	....	1.0
Riboflavin	0.5	1.0
a-Tocopherol	....	1.0
Pyridoxine hydrochloride	0.5	0.5
Thiamin chloride	0.3	0.5
Biotin	....	0.01

[1] Described by Schumacher and Heuser ('40).

[2] Bone meal — 2270 gm., K_2HPO_4 — 840 gm., pulverized limestone — 700 gm., NaCl — 600 gm., $MgSO \cdot 7H_2O$ — 500 gm., $Fe_2(SO_4)_2 \cdot XH_2O$ — 55 gm., $MnSO_4 \cdot 4H_2O$ — 29 gm., KI — 3.5 gm., $CuSO_4 \cdot 5H_2O$ — 1.5 gm., $ZnCl_2$ — 1.0 gm., $CoCl_2 \cdot 6H_2O$ — 0.2 gm.

[3] Phosphoric acid added to the extent of 0.2% of soybean oil as an antioxidant.

[4] Contained 400 A.O.A.C. units of vitamin D and 2000 U.S.P. units of vitamin A per gram.

The significance of the differences in growth response between the various treatments and between the three experiments was ascertained by means of analysis of variance (Snedecor, '34). No significant growth differences between the three experiments were found. This demonstrated that the methods of yeast extraction did not influence the distribution of growth activity in the various fractions, and that the data from the three experiments could be combined.

The results showed that fraction 1 promoted a growth response significantly greater than that of the unsupplemented control (odds>88:1), and that growth was not improved by increasing the amount fed. This growth-response plateau, significantly less than the response to yeast (odds>99:1), demonstrated that fraction 1 contains a growth factor (or factors) required by the chick, but is lacking in at

TABLE 2

Growth promoting effect of yeast fractions when added to simplified diet 543.[1]

Experiments 1, 2 and 3.

SUPPLEMENT	AVERAGE FOLIC ACID ADDED	NUMBER CHICKS 4 WEEKS	AVERAGE WEIGHT 4 WEEKS[2]
	µg./100 gm.		*gm.*
None	0.00	40	169
Fraction 1 ≎ 5% yeast	1.68	41	202
Fraction 1 ≎ 10% yeast	3.36	40	208
Fraction 1 ≎ 15% yeast	5.04	39	209
Fraction 2 ≎ 5% yeast	4.28	39	232
Fraction 2 ≎ 10% yeast	8.56	41	248
Fraction 2 ≎ 15% yeast	12.84	45	257
Fraction 3 ≎ 5% yeast	3.97	34	166
Fraction 3 ≎ 10% yeast	7.94	38	170
Fraction 3 ≎ 15% yeast	11.91	40	170
Fractions 1 + 2 ≎ 10% yeast	11.93	44	263
Fractions 2 + 3 ≎ 10% yeast	17.17	43	265
Fractions 1 + 3 ≎ 10% yeast	11.97	35	199
Fractions 1 + 2 + 3 ≎ 10% yeast	20.53	42	262
Yeast[3]	45.20	45	268

[1] Diet contained 26.7 µg. folic acid per 100 gm.

[2] Mean of male and female average weights.

[3] Ten per cent yeast used in two experiments. Eight per cent equal in growth-promoting effect to 10%, used in third experiment.

least one additional growth factor. These results agreed with those obtained by Schumacher, Heuser and Norris ('40) using a similar acid-alcohol precipitate fraction designated factor S. Therefore, fraction 1 contains the factor S of those workers.

Fraction 2 promoted a growth response significantly greater than that of fraction 1 (odds>99:1). As the amount of fraction 2 was increased, the growth response gradually increased until it approximated that promoted by yeast. Since fraction 1 supported only a submaximal

growth-response plateau, fraction 2 contained at least two factors because it was approximately equivalent to yeast in growth-promoting effect. These results differed from those obtained by Schumacher, Heuser and Norris ('40) since they obtained a submaximal growth-response plateau by feeding graded amounts of a neutral-alcohol precipitate prepared in a manner similar to fraction 2. This second factor they designated factor R. Therefore, fraction 2 contained factor R of Schumacher and associates ('40), but was contaminated with a small amount of factor S. Fraction 3, the alcohol-filtrate fraction, did not promote any significant growth response alone or in combination with any of the other fractions. In spite of the difference in growth response with factor R preparations, these experiments confirm the work of Schumacher and associates ('40) showing that at least two unidentified growth factors, factors R and S, are required by the chick.

In other work [4] with diet 543 factor R was freed of factor S by treatment of fraction 2 with Darco G-60. The adsorbed factor R, when graded levels were fed, was then found to give a submaximal growth-response plateau the same as in the work reported by Schumacher, Heuser and Norris ('40). Moreover, the filtrate remaining after treatment with charcoal was found to have a growth-promoting effect when fed at a level equivalent to 10% yeast but failed to supplement fraction 1 when supplied at the same level. Later with a purified diet it was found that fraction 2 is sometimes free of factor S before treatment with activated charcoal.

The folic acid content of the yeast fractions presented in table 2 was determined by the method of Mitchell and Snell ('41) using Streptococcus lactis R. The primary standard for the determinations was a partially purified folic acid preparation obtained from Dr. R. J. Williams of the University of Texas. It was stated to be 7.7% as pure as the material reported by Mitchell and Snell ('41). The standard for routine assays was a sample of liver fraction "B",[5] which was standardized against the folic acid concentrate. The samples were prepared for assay by a method similar to that of Cheldelin, Eppright, Snell and Guirard ('42).

The folic acid assay procedure of Mitchell and Snell ('41) was considered to give accurate results only in assays in which the folic acid concentrate and liver fraction "B" produced superimposable growth-response curves, and only when mixtures of these two materials promoted the same growth response as an equivalent amount of folic acid

[4] Unpublished results.

[5] The Wilson Laboratories, Chicago, Illinois.

from either one alone. The galvanometer readings falling outside the straight-line portion of the standard growth curve were not used in establishing folic acid content. This procedure has been found recently at this laboratory [6] to give results in agreement with those obtained by the revised assay procedure reported by Luckey, Briggs and Elvehjem ('44).

The folic acid values presented in the tables of this paper are expressed as micrograms of folic acid of a potency 40,000 times that of the liver fraction "B" used by the University of Texas workers. Under these conditions the liver fraction "B" contained 17.9 μg. of folic acid per gram.

In this work the same type of growth-response curve was obtained with Streptococcus lactis, when pure crystalline Lactobacillus casei factor prepared from liver by E. L. R. Stokstad [7] was used as a standard, as when Williams' folic acid concentrate or when liver fraction "B" was used. No direct relation was found to exist between the folic acid content of the yeast fractions and their growth promoting activity for the chick. Fraction 3 which contained more folic acid than fraction 1 promoted no growth-response alone or in combination with the other fractions. Therefore, neither factor R nor factor S is identical with folic acid, and the added folic acid, if required, is ineffective in promoting growth in the absence of both factor R and factor S.

However, the folic acid content of simplified diet 543 (26.7 μg. per 100 gm.) was probably adequate to meet the requirements of the chick for this substance. Data obtained in other experiments [8] conducted at this laboratory showed that two appropriately supplemented purified diets, containing 15.2 and 20.0 μg. of folic acid per 100 gm., supported maximum growth under the experimental conditions used. The growth-response in these experiments was not increased by the addition of more folic acid in the form of yeast to the diets.

The report of Pfiffner and associates ('43) that pure preparations of the chick antianemia factor, vitamin B_c, are active in stimulating the growth of Lactobacillus casei ε and are probably identical with folic acid, made it of interest to determine if chicks fed simplified diet 543 develop anemia. The results showed that the average blood hemoglobin values (9.8 and 9.3 mg. per 100 ml.) of chicks fed the unsupplemented diet were normal and approximately the same as those (9.3 and 9.6 mg. per 100 ml.) of chicks receiving this diet supplemented with yeast. They

[6] See footnote 4, p. 176.
[7] Lederle Laboratories, Pearl River, New York.
[8] See footnote 4, p. 176.

demonstrated that simplified diet 543 used in these studies was not lacking in any antianemic factor, and that neither factor R or S is identical with vitamin B_c unless more of it is required for maximum growth than for the prevention of anemia.

Experiments with purified diet 653

In order to investigate the properties of factors R and S under more rigorous conditions, studies were conducted using purified diet 653. The composition of the diet is given in table 1. The inclusion of either cystine or calcium gluconate in the diet has been found not to improve chick growth at this laboratory.[9] Nutritional encephalomalacia, however, was obtained when calcium gluconate was not used. Further difficulty of this character was prevented by adding 0.2% of orthophosphoric acid to the soybean oil in the diet. Whenever this diet was fed alone, growth failure, severe anemia and extreme mortality occurred.

The yeast fractions were prepared from water extracts of yeast in accordance with the procedure described previously. An adsorbate of fraction 2 (fraction 2-A) was fed in these experiments, as well as fraction 1 and fraction 2. A quantity of fraction 2 equivalent to 1 kilogram of yeast was dissolved in 1 liter of water and digested at pH 4.6 with 2 gm. of takadiastase for 24 hours at room temperature. The mixture was brought to pH 7.0 with NaOH and steamed 30 minutes in an autoclave. Upon cooling a flocculent white precipitate formed. This was removed by filtration, dissolved in warm 5% sulfuric acid and reprecipitated with excess ammonia. The filtrate from this precipitation was combined with the original filtrate and brought to pH 3.0 with sulfuric acid. Five gm. of heat-activated Darco G-60 were added to this mixture. It was then stirred for 30 minutes and filtered.

In the first experiment fraction 2 was fed as a supplement to diet 653 at levels equivalent to 5, 10 and 15% of yeast. Fraction 2-A was also fed at these levels. Each lot contained fifteen White Leghorn male chicks. The results are given in table 3, experiment 4.

The results showed that growth-response plateaus were obtained with levels of fraction 2 and fraction 2-A equivalent to 10% yeast. In both instances the plateaus were significantly lower than the growth-response obtained with yeast. Therefore fraction 2 and fraction 2-A contained a factor (factor R) required for growth and lacked another factor required along with factor R for maximum growth.

[9] See footnote 4, p. 176.

TABLE 3

Growth promoting effect of yeast fractions when added to purified diet 653.[1]

SUPPLEMENT	FOLIC ACID ADDED	NUMBER CHICKS 4 WEEKS	AVERAGE WEIGHT 4 WEEKS	NUMBER CHICKS 6 WEEKS	AVERAGE WEIGHT 6 WEEKS	BLOOD HEMOGLOBIN 5 WEEKS
	μg./100 gm.		*gm.*		*gm.*	*gm./100 ml.*
Experiment 4						
None	0.0	3	92	0	...	4.3 (7)[2]
Fraction 2 ⊂⊃ 5% yeast	5.0	13	173	11	245	7.2 (11)
Fraction 2 ⊂⊃ 10% yeast	10.0	15	248	15	440	8.9 (15)
Fraction 2 ⊂⊃ 15% yeast	15.0	15	243	13	434	9.6 (13)
Fraction 2-A[3] ⊂⊃ 5% yeast	5.0	13	139	7	200	5.4 (8)
Fraction 2-A ⊂⊃ 10% yeast	10.0	13	241	12	424	7.9 (12)
Fraction 2-A ⊂⊃ 15% yeast	15.0	14	252	14	438	9.2 (14)
10% yeast	48.4	15	312	14	548	9.9 (15)
Experiment 5						
None	0.0	7	112	1	150	3.5 (5)
Fraction 1 ⊂⊃ 5% yeast	4.6	9	137	4	214	5.5 (6)
Fraction 1 ⊂⊃ 10% yeast	9.2	12	174	9	245	5.1 (11)
Fraction 1 ⊂⊃ 15% yeast	13.8	14	199	13	261	6.5 (13)
Fraction 2 ⊂⊃ 15% yeast	15.0	15	206	15	379	9.0 (15)
Fraction 1 + fraction 2 ⊂⊃ 10% yeast	19.2	15	244	13	491	9.8 (13)
Fraction 2-A ⊂⊃ 15% yeast	15.0	14	214	14	361	6.8 (14)
Fraction 2-A + fraction 1 ⊂⊃ 10% yeast	19.2	14	255	14	493	9.0 (14)
Fraction 3 ⊂⊃ 10% yeast[4]	7.7	4	100	0	...	4.8 (2)
10% yeast	48.4	15	279	14	488	9.2 (14)
Experiment 6[5]						
None	0.0	8	111	1	130	4.6 (3)
Fraction 2-A ⊂⊃ 10% yeast	5.0	10	173	9	276	9.5 (9)
Fraction 3-A ⊂⊃ 10% yeast[6]	6.5	6	108	0	...	4.7 (2)

[1] Diet 653 contained 8.9 μg. folic acid per 100 gm.

[2] Hemoglobin at 26 days. Figure in parentheses indicates number of chicks.

[3] Dareo adsorbate prepared from fraction 2.

[4] Also contained fraction 1-F, fraction 1 freed of folic acid and the antianemic factor by treatment with Dareo.

[5] Each lot contained 10 White Leghorn male chicks at start. Chicks fed a modification of diet 653, containing glucose in place of corn starch, somewhat less casein and gelatin, and 0.5% cystine. The modified diet contained 7.8 μg. folic acid per 100 gm.

[6] Dareo adsorbate prepared from fraction 3.

A further study was conducted to determine the supplementary action of factor S present in fraction 1, and factor R present in fractions 2 and 2-A when included in purified diet 653. Fraction 1 was fed at levels equivalent to 5, 10 and 15% of yeast. The same preparations of fractions 2 and 2-A, used in experiment 4, were fed at levels equivalent to 15% of yeast, in order to establish the maximum growth-responses of the chicks used in this experiment to these materials. Fractions 2 and 2-A were also fed in combination with fraction 1 at levels equivalent to 10% of yeast. Each lot contained fifteen White Leghorn male chicks. The results are given in table 3, experiment 5.

The results showed that fraction 1 produced a growth response which tended to give a plateau at a submaximal level as in previous work. Fractions 2 and 2-A promoted submaximal growth which, in view of the results of experiment 4, represented the maximum responses to these materials. The combination of fraction 1 with fraction 2 or fraction 2-A promoted maximum growth equal to that promoted by yeast. The plateau and supplementary effects obtained in this experiment and the preceding one showed that fraction 1 contains a factor (factor S) which is not identical with factor R present in fraction 2.

The results of the experiments with purified diet 653 confirm the results obtained with simplified diet 543 and demonstrate the existence of two unknown factors, designated factors R and S, which are required for chick growth. Neither one of these factors is choline, since purified diet 653 was adequately supplemented with choline. Also neither one is identical with folic acid since both fraction 1 and fraction 2 promoted submaximal growth-response plateaus upon feeding graded levels in spite of the presence in them of approximately the same quantities of folic acid. If one of the factors had been folic acid, a submaximal plateau would have been revealed in one instance only.

In these experiments both fraction 1 and fraction 2 were found to possess antianemic properties. Fraction 1, however, was much less effective in preventing anemia than fraction 2 in spite of the fact that it contained almost as much folic acid as this fraction. The addition of 13.8 μg. of folic acid per 100 gm. of diet 653 by means of fraction 1 maintained a hemoglobin level of only 6.5 gm. per 100 ml. of blood as compared to a level of 8.9 gm. per 100 ml. maintained by the addition of 10 μg. of folic acid by means of fraction 2. Fraction 1, therefore, is deficient in an antianemic factor present in fraction 2, and this antianemic factor is not folic acid. The somewhat higher hemoglobin level maintained by fraction 1 as compared to the unsupplemented purified diet probably was due, in part at least, to contamination of factor S

with this antianemic factor, since preliminary results showed that Darco treated fraction 1 alone possessed no antianemic properties. It was not entirely due to folic acid since in experiment 5 the addition of fraction 3, which contains folic acid but no factor R or S, to the purified diet failed to increase the hemoglobin level. The same results were obtained in experiment 6 with fraction 3-A, the adsorbate of fraction 3. On the other hand, fraction 2-A, the adsorbate of fraction 2, which contained less folic acid in this experiment than fraction 3-A promoted normal hemoglobin formation.

The fraction 2 and fraction 2-A, used in experiments 4 and 5, were somewhat more effective in promoting growth than in preventing anemia. Both fractions at levels equivalent to 10% yeast promoted growth as great as that obtained at the 15% levels but the 15% levels of these fractions were better than the 10% levels in preventing anemia. Furthermore, in both experiments the adsorbate of fraction 2 appeared somewhat less effective in promoting hemoglobin formation than fraction 2 but no evidence was obtained of an accompanying reduction in growth promoting effect. This decrease in antianemic effect was particularly striking in experiment 5 where chicks of somewhat lower inherent growth capacity were used. At times, however, these differences were not observed, as in experiment 6, when the yeast extract used in preparing the fraction was richer in the antianemic factor than that from which the fractions studied in experiments 4 and 5 were prepared. In spite of this the results of the experimental work with purified diet 653 indicate that the antianemic factor is not identical with factor R, and that it is either somewhat less readily adsorbed by activated charcoal or less readily eluted by the chick than the growth factor. It is evident also that the antianemic factor is not identical with factor S.

These conclusions are strengthened by comparing the results obtained with purified diet 653 with those obtained with simplified diet 543. The latter diet was found to be deficient in factors R and S but not in the antianemic factor or folic acid. Purified diet 653, on the other hand, was found to be lacking in factors R and S and the antianemic factor. It also may be deficient in folic acid. With simplified diet 543 both factor R and factor S were shown not to be identical with the antianemic factor unless more of these factors are required for maximum growth under the experimental conditions than for the prevention of anemia. This is impossible since the work with purified diet 653 demonstrated that the antianemic factor accompanies factor R for the most part and that in some instances more of fraction 2 and fraction 2-A which contain factor R was required to prevent anemia under the experimental conditions than to promote maximum growth.

DISCUSSION

According to the work of Mitchell, Snell and Williams ('41), Hutchings, Bohonos and Peterson ('41), Stokstad ('43), and Mitchell and Williams ('44), folic acid and the Lactobacillus casei factor appear to be identical. The work of Pfiffner and associates ('43) and Stokstad ('43) provides evidence that the Lactobacillus casei factor and vitamin B_c are also identical. If those factors are the same, the antianemic factor encountered in the experiments reported in this paper is a new factor, differing from the substance variously designated folic acid, Lactobacillus casei factor and vitamin B_c.

The work conducted with purified diet 653, however, does not preclude the possibility that folic acid is a factor required by the chick both for growth and the prevention of anemia. When fraction 3 or fraction 3-A only were added to purified diet 653 the hemoglobin level was not increased. Growth failure and 100% mortality also occurred as was observed in several instances with the purified diet alone. However, folic acid may function as an antianemic and growth factor only in the presence of the antianemic factor found chiefly in fraction 2. The existence of a second antianemic factor is suggested by the work of O'Dell and Hogan ('43). If this is true, the folic acid requirement of the chick for growth and the prevention of anemia appears to be less than 15 μg. per 100 gm. of diet. Briggs, Luckey, Elvehjem and Hart ('43) have reported maximum growth in chicks fed a purified diet containing 17.5 μg. of added folic acid per 100 gm. The folic acid content of the purified diet was not given but, since it was similar to purified diet 653, it probably contained approximately 8 μg. per 100 gm.

The evidence that the chick requires a second antianemic factor in addition to folic acid, however, is difficult to reconcile with the work of Pfiffner and associates ('43) and Campbell, Brown and Emmett ('44). Under their experimental conditions, these workers obtained maximum growth in chicks and prevention of anemia by supplying from 100 to 400 μg. of vitamin B_c per 100 gm. of diet. The antianemic effect obtained by Campbell and associates ('44), however, was not as striking as that obtained by Pfiffner and associates ('43) and may have been subnormal. In contrast to these growth results, Almquist ('43) failed to obtain any significant growth increase by supplying 150 μg. of Lactobacillus casei factor per 100 gm. of diet by means of a concentrate, approximately 9% pure, prepared by Stokstad.[10]

In view of these results and in the light of the studies reported in this paper showing that the folic acid requirement of the chick is less

[10] See footnote 7, p. 180.

than 15 μg. per 100 gm. of diet, it is suggested that the antianemic effect of crystalline vitamin B_c and also its growth promoting properties are caused either by contamination with extremely potent antianemic and growth factors or to synthesis of these factors by bacteria in the intestinal tract as a consequence of supplying large amounts of a necessary bacterial growth factor. O'Dell and Hogan ('43) showed that the incidence of anemia is increased by including sulfaguanidine in the purified diet fed the chicks. If either of these explanations proves to be correct, then the antianemic factor encountered in the studies described in this report is probably the vitamin originally discovered by Hogan and Parrott ('40) and designated vitamin B_c.

In view of the ease of adsorption by activated charcoal and because of similarities in source and function, factor R may be identical with factor U of Stokstad and Manning ('38). Vitamin B_{11} of Briggs, Luckey, Elvehjem and Hart ('43) appears to be the same as factor R of Schumacher, Heuser and Norris ('40) but is associated with an antianemic factor. The other unidentified factor suggested by Briggs and associates ('43) as a consequence of failure to obtain as good growth with a biotin concentrate as with kidney residue may be identical with factor S of Schumacher and associates ('40).

No evidence of the existence or non-existence of a factor essential for feather development only was obtained in the experiments reported in this paper. Factor S, however, did not promote as good feather development as factor R. Since the results of Briggs, Luckey, Elvehjem and Hart ('43) indicate that there is a feather factor distinct from vitamin B_{11}, it may accompany factor R in fraction 2.

SUMMARY

Evidence, obtained previously, of the existence of two unidentified chick growth factors, designated factor R and factor S, has been confirmed. Factors R and S have been found not to be identical with folic acid, the Lactobacillus casei factor or vitamin B_c. If the latter factors are the same substance, as recent work indicates, a new chick antianemic factor has been revealed which is distinct from factors R and S. This antianemic factor may be vitamin B_c, however, in the event that crystalline preparations of it are contaminated with highly potent growth and antianemic factors or stimulate their bacterial synthesis in the intestinal tract. No evidence was obtained that folic acid is, or is not, required by the chick, since growth failure and very extreme mortality occurred in the absence of the antianemic factor and factor R or

the antianemic factor and factor S. If folic acid is required, the amount appears to be less than 15 μg. per 100 gm. of diet.

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PROTEIN INTAKE AND HEAT PRODUCTION[1]

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During recent years the authors and their associates have conducted six experiments, five with growing and one with mature albino rats, for the purpose of determining the influence of the protein content of equicaloric diets on the heat production under conditions representing normal nutritive practice; and in these experiments the heat production diminished, at moderate rates, in the increasing order of the intake of protein — which is not in harmony with the generally prevailing understanding that the protein content of diets dominates the heat production by virtue of the high specific dynamic effect of this nutrient as fed alone. Five of these experiments have been published in four papers (Forbes, Swift, Black and Kahlenberg, '35; Forbes, Voris, Bratzler and Wainio, '38; Forbes, Black, Thacker and Swift, '39, '40), and the results of the sixth are presented herewith. In this general program 231 albino rats were used as subjects, with 10 to 12 animals on each dietary treatment, the program as a whole covering 16,170 24-hour "rat days" of controlled experimentation.

In this series of experiments the heat production was measured as single values for 70-day periods by subtracting the caloric equivalents of the excreta and of the body gain from the gross energy of the food. The special advantages of this procedure, which the authors have called "the body balance method", are that the long period of observation serves to minimize the factor of error of work, and covers the complete process of food utilization during a protracted interval. Also, in order that the results shall represent the normal use of food, the animals were fed at super-maintenance planes of nutrition on diets which were nutritively complete, except as influenced by the protein intake, and were allowed unrestricted freedom of motion. This procedure has led to conclusions at variance with the generally prevailing ideas as to the influence of the protein intake on the production of heat because of

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differences in methods of experimentation, and in the interpretation of results, as will later appear.

To assist in clarifying our understanding of the influence of protein on the heat production from normal, mixed diets the authors conducted the experiment discussed in this paper. The general procedure was the same as in the previous experiments of this series, except that the environmental temperature for the animals was maintained during the entire 70 days of the experiment definitely within the zone of thermal neutrality, that is, within the range of temperature at which no extra nutriment is katabolized for the maintenance of the normal temperature of the body. During the earlier experiments of this series the environmental temperatures were not so controlled and were often slightly below the critical point, though all groups of animals received the same treatment.

The idea that the dynamic effects of normal diets are in harmony with their protein contents depends primarily on two misconceptions, (1) that specific dynamic effects of nutrients determined at planes of nutrition involving the katabolism of body substance apply to supermaintenance planes of nutrition, and (2) that the specific dynamic effects of individual nutrients, determined by any procedure, are true of these nutrients when combined as in the diets of normal nutrition.

The usual method of determining the specific dynamic effects of nutrients is to give a single feeding of the test substance to an animal in a post-absorptive state and then, during intervals of time in which the subject is quiet, to measure the maximum height of the increase in heat production, or more properly the total increase in heat production above the base rate, or above a mean value between the initial base rate and a final rate computed to represent the decreased heat production of the subject if it had continued without food during a time interval equal to the period of measurement of the increased metabolism.

The element of experimental error in any such short-time measurements is apt to be large, especially as the result of a technic inadequate for the determination of either the maximum height of the metabolism attained or the total increase in heat production; and it is the authors' understanding that any such measurement, involving the katabolism of body substance, is too low to apply to planes of nutrition at or above maintenance by an amount representing the dynamic effect of the body nutrients katabolized. The point of view from which this observation is made has already been discussed (Forbes and Swift, '41).

That determinations of dynamic effects by procedures inplying the heat production at a sub-maintenance status as a base value do not

apply to normal nutrition at super-maintenance rates is also indicated by the fact that the proportion in which the nutrients are utilized is much more prominently affected when the base value is below than when it is at or above maintenance. Also, the assumption that the specific dynamic effects of individual nutrients as determined by any procedure apply to combinations of nutrients is not in accord with established facts.

In connection with the presentation of evidence on the heat increments of diets balanced and unbalanced with respect to protein, Hamilton ('35, '37, '39) has summarized abundant literature showing that nutritive balance, in general, affects the economy of utilization of food energy; and an extensive series of papers from this institute, by E. B. Forbes and associates, on individual nutritive deficiencies as affecting the utilization of food energy, similarly imply that the heat increments and the energy values of nutrients in combination can not be the additive separate heat increments and energy values of these constituent nutrients.

Further, Forbes, Bratzler and associates ('39), Ring ('42), and Forbes and Swift ('44) have conducted experiments showing that the dynamic effects of protein, carbohydrate and fat as individually determined are not true of these nutrients variously combined.

EXPERIMENTAL

In the present experiment the plan of the diets as given in table 1 shows that their protein contents were determined by the proportions of beef muscle protein included; and the gross energy values of the diets were rendered equal by compensating adjustment of their contents of carbohydrate [2] and fat.[3] As a result, the arrangement of the diets in the order of their increasing protein contents had the effect of arranging them in the order of their decreasing contents of carbohydrate and fat. Critically speaking, therefore, the differences in the results produced by these diets should be ascribed not to the differences in protein intake alone but to this factor and the associated, compensating differences in carbohydrate and fat.

Referring to table 2 — the subjects of this experiment were eleven litter-triplets of weanling, male, rats divided into three groups of approximately the same weight, which received equicaloric quantities of diets containing approximately 10%, 25%, and 45% of protein. All results of this experiment, therefore, are averages representing eleven animals.

[2] Cerelose. [3] Crisco.

Since the 25% protein diet was more nearly the optimum than the other two, for the growing rat, the gains in live weight and in nitrogen produced by this diet were greater than by the diets of 10% and 45% protein; but the gain in fat decreased in the increasing order of their contents of protein, which was in the decreasing order of their contents of metabolizable energy.

TABLE 1

Composition of diets.

INGREDIENT	DIET 1	DIET 2	DIET 3
	%	%	%
Cellu flour	4.00	4.00	4.00
Salt mixture [1]	4.00	4.00	4.00
NaCl	1.00	1.00	1.00
Yeast [2]	6.00	6.00	6.00
Butterfat	1.00	1.00	1.00
Cerelose [3]	55.60	43.45	27.26
Beef muscle	9.20	29.58	56.74
Crisco	19.20	10.97	0.00
Total	100.00	100.00	100.00

[1] Osborne, T. B., and L. B. Mendel, '17. J. Biol. Chem., vol. 32, p. 369.

[2] The yeast used was a mixture of 5 parts by weight brewer's yeast and 1 part irradiated yeast.

[3] Cerelose is a crystalline dextrose of high purity.

TABLE 2

Average amounts of food eaten, and of gains in live weight, nitrogen and fat per rat during 70 days.

PROTEIN IN DIET	FOOD EATEN, DRY MATTER	LIVE WEIGHT		GAIN IN EMPTY BODY WEIGHT	NITROGEN OF BODY GAIN	FAT GAINED
		Initial	Final			
%	gm.	gm.	gm.	gm.	gm.	gm.
10.06	498.4	33.5	146.2	108.1	3.02	21.68
24.81	499.9	33.1	201.5	161.7	5.38	19.01
44.88	499.8	33.3	180.2	141.1	4.76	16.43

The distribution of the total food nitrogen per rat, for 70 days, is given in table 3; and the distribution of the food energy for the same period is presented, as from experiment no. VI in table 4, which gives parallel data from the entire series of six similar experiments conducted at this laboratory.

The protein variant in experiments I, II and III was casein; and in experiments IV, V and VI it was beef muscle protein.

As revealed by their live weights, the rats in experiments I, II, III, IV and VI were weanlings, in contrast to those in experiment V which

were mature. The lightness of the initial live weights of the rats in experiment VI was due to a nutritive deficiency resulting from war-time restrictions affecting the manufacture of the commercial animal feed which served as the principal part of the stock colony ration.

In this table the data representing "metabolized energy" are the values for food energy minus the energy of the feces and urine, and those for "metabolizable energy" are the values for food energy minus the energy of the excreta and the non-metabolizable energy of the protein retained.

TABLE 3

Distribution of average 70 days' food nitrogen per rat as related to the plane of protein intake.

Protein in the diet, %	10.06	24.81	44.88
Nitrogen:			
Food, gm.	8.5	21.0	38.0
Feces, gm.	1.0	1.6	2.7
Digested, gm.	7.5	19.4	35.3
% of food N	88.2	92.4	92.9
Urine, gm.	4.4	13.9	29.4
% of food N	51.8	66.2	77.4
Gain of N, gm.	3.0	5.4	4.8
Calories in urine per gm. urinary N	11.7	8.0	7.0

CONCLUSIONS

From a series of six experiments, five of which have been previously published, on the utilization of the energy and the protein of equicaloric diets differing in protein content, the following conclusions are drawn:

The apparent digestibility of food nitrogen, and the per cent of the food nitrogen eliminated in the urine increased, and the energy per gram of nitrogen in the urine decreased, in the increasing order of the protein contents of the diets.

The apparently digestible energy, as indicated in reverse by the energy of the feces, increased in the increasing order of the protein contents of the diets at the lower planes of protein intake, but in three experiments among the four in which the diet contained 45% of protein, the plane of maximum apparent digestibility of protein was exceeded.

In all experiments the outgo of energy in the urine increased throughout the entire range of increase in protein intake.

The body gain of energy increased in the increasing order of the protein contents of the diets from 10% to 25%, but decreased in the order of the further increase in protein content of the diets to 45%.

TABLE 4

Distribution of average 70-day food energy of albino rats related to the percentage of protein in equicaloric diets.

EXPERIMENT NO. AND REFERENCE TO PUBLICATION	LIVE WEIGHTS OF RATS	PROTEIN IN DIETS, APPROXIMATE PERCENTAGE	FOOD ENERGY PER RAT	FECES ENERGY	URINE ENERGY	BODY GAIN OF ENERGY	BODY GAIN OF PROTEIN ENERGY	BODY GAIN OF FAT ENERGY	METABOLIZED ENERGY [1]	METABOLIZABLE ENERGY [1]	HEAT PRODUCTION
	gm.	*%*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*
I	48–126	10	2164	184	47	224	93	131	1933	1914	1709
J. Nutr. 10	48–155	15	2164	178	55	263	137	125	1931	1902	1668
(1935), 461	48–168	20	2164	153	72	296	153	143	1939	1907	1643
	47–167	25	2164	143	93	302	156	145	1928	1895	1626
II	48–115	10	2076	178	39	226	68	158	1858	1844	1632
J. Nutr. 10	47–142	15	2076	162	54	259	108	152	1860	1837	1601
(1935), 461	48–150	20	2076	148	71	267	117	150	1857	1833	1590
	48–152	25	2076	138	90	270	121	149	1848	1823	1578
III	47–162	25	2261	161	99	332	129	203	2001	1974	1669
J. Nutr. 15	47–159	30	2261	156	121	320	127	193	1984	1957	1665
(1938), 285	48–159	35	2261	149	142	311	127	184	1970	1943	1658
	47–151	45	2261	140	187	285	119	166	1934	1909	1650
IV	49–173	10	3136	339	58	364	136	228	2739	2711	2375
J. Nutr. 18	50–218	25	3136	296	142	480	201	279	2698	2656	2218
(1939), 47	50–220	35	3136	279	209	423	208	215	2648	2605	2225
	50–207	45	3136	298	261	415	189	226	2577	2537	2162
V	385–391	10	4220	498	103	113	9	104	3619	3617	3505
J. Nutr. 20	388–401	25	4220	403	237	124	14	110	3579	3576	3456
(1940), 47	390–388	45	4220	413	401	5	12	−6	3407	3404	3402
VI	34–146	10	2505	275	51	323	116	207	2179	2155	1856
J. Nutr.,	33–202	25	2505	237	111	387	207	180	2157	2114	1770
(this paper)	33–180	45	2505	271	206	334	179	155	2028	1991	1694

[1] For a statement of the distinction between metabolized and metabolizable energy see the text.

The body gain of protein energy increased in accord with the increasing protein intake up to the 30% to 35% level, but was less extensive at the 45% than at the 35% level.

The body gains of fat energy represented balances of metabolizable energy supplied in excess of the requirements of the animal for maintenance (including physical activity) and synthesis of protein. The gains in fat energy were not in definite order with reference to the plane of protein intake.

The metabolized energy was about on a level at planes of protein intake up to 25%, but invariably decreased at the higher levels of dietary protein.

The metabolized energy, the metabolizable energy, and the heat production all diminished at about the same slight rate throughout the entire range of increase in the protein contents of the equicaloric diets, thus rendering clear the fact that it was the metabolizable energy, and not the protein content of these diets, which dominated the production of heat.

The improved condition of experimentation provided in the present study as compared with the previous studies of the series — that is, the maintenance of the environmental temperature within the zone of thermal neutrality for the subjects — was without observed effect on the results obtained.

SUMMARY

Results are presented of a study of the heat production of growing albino rats in relation to the protein contents of equicaloric diets containing approximately 10%, 25% and 45% of protein; and general conclusions are drawn from six similar studies, including the present experiment, on the same subject.

The special purpose of this last experiment was to determine the effects of maintaining the environment at all times definitely within the zone of thermal neutrality for the subjects. This improvement in conditions of experimentation was without observed effect on the results obtained, as compared with previous findings.

The metabolizable energy and the heat production diminished at about the same slight rate throughout the entire range of increase in the protein contents of the equicaloric diets, thus showing that it was the metabolizable energy, and not the protein content of these diets, which dominated the production of heat.

Other conclusions are given in the text.

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INTESTINAL ABSORPTION OF GALACTOSE IN THE RAT AS AFFECTED BY SUBOPTIMAL INTAKES OF THIAMINE [1, 2, 3]

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ONE FIGURE

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A decrease in the rate of intestinal absorption of galactose in acute severe thiamine deficiency and in vitamin B complex deficiency has already been reported by this laboratory (Free and Leonards, '42; Leonards and Free, '43b). The possibility that prolonged subacute thiamine deficiency, which is more prevalent than the acute severe deficiency, may have an effect on intestinal absorption led to the present study. Specifically, it was designed to determine the effect of prolonged subsistence on diets low in thiamine on the rate of intestinal absorption of galactose in the rat.

METHODS

The methods used in the care of animals and in the measurement of intestinal absorption have already been adequately described (Free and Leonards, '42). Three groups of young rats were placed on the vitamin B complex-free diet having the following percentage composition: alcohol-extracted casein 18, starch 68, cottonseed oil 8, Mendel-Hubbell-Wakeman salt mixture ('37), 4, and cod liver oil 2; to 100 parts of this mixture was added 0.2 part of choline chloride. One group of rats received this diet supplemented with 2 μg. of thiamine daily, the second group with 5 μg. daily and the third group with 10

[1] The material contained in this paper is taken from a dissertation submitted by Jack R. Leonards, to the Graduate School of Western Reserve University, June 1943, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry. The authors are indebted to Prof. Victor C. Myers for making possible the series of investigations on the effect of vitamin deficiencies on intestinal absorption.

[2] This investigation was supported by a grant from the Williams-Waterman Fund of the Research Corporation, New York.

[3] We are indebted to Merck and Co., Rahway, New Jersey, for generous supplies of the vitamins employed in this study.

μg. per day. Each experimental animal was "paired" with a littermate control rat which received 40 μg. of thiamine per day, and whose food intake was limited so that its weight was kept very nearly equal to that of the experimental member of the pair.

All of the rats received the usual adequate daily supplements of riboflavin (25 μg.) pyridoxine (20 μg.), and calcium pantothenate (100 μg.). The animals were kept on this diet for 70 to 80 days at which time the weights of animals receiving the 2 μg. and 5 μg. of thiamine per day had become stabilized. At this time the measurements of intestinal absorption of galactose were carried out.

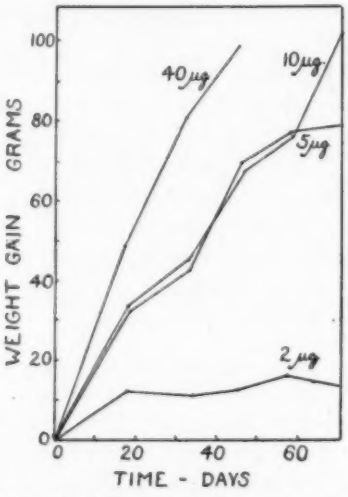

Fig. 1 The effect of various thiamine supplements on the rate of growth of rats.

RESULTS

The average growth curves of the rats fed diets containing 2 μg., 5 μg. and 10 μg., respectively, of thiamine daily are given in figure 1, and are compared with those of a group of rats on the same diet eaten ad libitum but supplemented with 40 μg. of thiamine daily. These growth curves do not include the weights of the paired control animals, but as previously mentioned the weights of these controls were kept very nearly the same as those of the experimental companions. The animals receiving 5 μg. and 10 μg. of thiamine daily grew at about the same rate for the first 8 weeks after which time the 5 μg. group did not gain any more weight. Two μg. of thiamine daily was sufficient to enable the rats to attain a weight of about 75 gm. The weights then fluctuated

within 5 or 10 gm. of this figure for the last 50 days of the experiment. Thus the condition of most of the animals in this group was kept rather constant for a considerable period of time. Four of the animals receiving 2 μg. of thiamine per day exhibited marked symtoms of polyneuritis after being on the diet for about 60 days. These were promptly cured by allowing the rats to drink a solution containing 20 μg. of thiamine. Then, in order to prevent the rats from gaining too much weight, the regular daily thiamine supplements were withheld for 5 or 6 days. This procedure was thought to be preferable to attempting to cure the rats with smaller doses of thiamine. No recurrences of polyneuritis were observed in these rats.

The animals receiving the higher levels of thiamine appeared normal in all respects, except for a slight decrease in the rate of growth. The rats on the 2 μg. daily level of thiamine, as well as their pair-fed controls, appeared emaciated and had rough fur. Priapism was observed in many of the animals both in the deficient and control groups. Although care was taken to assure an adequate supply of pantothenic acid, about one-half of the animals in the 2 μg. level group and their controls showed a "rusting" of the white fur around the neck, similar to that seen in pantothenic acid deficiency (McElroy, Salomon, Figge and Cowgill, '41). This was only of a very mild degree, and there were no "blood caked" whiskers.

The individual results of the absorption studies of 25 pairs of rats in which the experimental member was allowed 2 μg. of thiamine daily are given in table 1. The average absorption coefficient of the deficient animals was 298 mg. per 100 gm. per hour while that of the controls averaged 354 mg. per 100 gm. per hour. The rate of absorption of the deficient animals therefore averages 85% of that of the controls. This decrease in rate of absorption of the chronically deficient animals, although small and at the borderline of statistical significance, is exhibited by twenty-one of the twenty-five animals that were studied. That this decrease is not due mainly to changes in the factors of gastric motility and emptying time is shown by the fact that the intestines of the deficient animals contained even more galactose than those of the controls. The values are, respectively, 11% and 7% of the ingested amount of the sugar.

The average values for the blood galactose 1 hour after ingestion of the sugar were respectively, 513 mg. per 100 ml. and 578 mg. per 100 ml. for deficient and control groups. Thus the rate of intestinal absorption of the rats on the low thiamine intake, as measured by the blood galactose levels, averaged 89% of that of the control animals.

The blood galactose levels therefore do not indicate any impairment in the rate of metabolism of the sugar as was seen in the case of rats acutely deficient in pantothenic acid and thiamine (Leonards and Free, '43a; Leonards and Free, '43b).

The results obtained on the effects of daily intakes of 5 μg. and 10 μg. of thiamine daily on the rate of intestinal absorption of galactose are also given in table 1. There is no significant difference in the rate of intestinal absorption in rats receiving 5 μg., 10 μg., or 40 μg. respectively, of thiamine daily. There are, however, two interesting observations in these animals to be reported. One is that the absorption

TABLE 1

Intestinal absorption in rats receiving different thiamine supplements.

	NUMBER OF RATS	BODY WEIGHT		GALACTOSE				DIFFERENCE CONTROL MINUS DEFICIENT	
		Initial	Final	In stomach[1]	In intestines[1]	Absorption coefficient	Blood galactose	Absorption coefficient	Blood galactose
		gm.	*gm.*	%	%	*mg./100 gm./hr.*	*ml.*		
2 μg.[2]	25	56	71	39	11	298	513		
Control	25	47	74	34	7	354	578	+ 52	+ 61
5 μg.	7	45	115	57	10	198	381		
Control	7	39	105	53	7	236	472	+ 38	+ 89
10 μg.	8	33	129	53	8	233	444		
Control	8	31	123	49	8	256	478	+ 24	+ 35

[1] Fraction of ingested galactose

[2] Daily thiamine supplement.

coefficients of these groups of animals are much lower than those obtained for all of the other groups of rats in vitamin deficient states which have been studied in this laboratory. The other observation is that the per cent of ingested galactose remaining in the intestines of these rats was also lower than that characteristic of other groups of animals, the amount of sugar in the stomach being correspondingly increased. One reason for these differences may be due to the fact that the growth of these animals was not stunted as much as in the more severe deficiencies.

Harper ('42) studied the effect of suboptimal intake of thiamine on the rate of intestinal absorption in rats. The rats were given a thia-

mine deficient diet for 6 weeks or until their food consumption diminished. They were then given 10 μg. of thiamine daily for a period which was not recorded. A decrease in the rate of absorption of glucose was observed in the deficient animals. This decrease amounted to 17% of the normal rate when a 2-hour absorption period was used. The effect of gastric evacuation was not recorded. Although the conditions of Harper's experiments were different than those described in this report, both studies indicate that only small decreases in the rate of intestinal absorption of sugars are observed in mild chronic thiamine deficiency.

SUMMARY

1. The rate of intestinal absorption of galactose was measured in 3 groups of rats which were kept for 70 to 80 days on thiamine intakes of 2 μg., 5 μg., and 10 μg. per day, respectively. The absorption was compared with "paired" control animals receiving 40 μg. of thiamine per day.

2. The rate of absorption of galactose in rats receiving these suboptimal thiamine intakes averaged 85% to 90% of that of the controls.

3. The methods employed did not indicate any change in the rate of metabolism of galactose as a result of the chronic thiamine deficiency.

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BIOTIN AND FOLIC ACID DEFICIENCIES IN THE MOUSE

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ONE PLATE (FOUR FIGURES)

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Rats maintained on a purified diet containing the B Complex vitamins — thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid and choline chloride — grow normally (Unna, Richards, and Sampson, '41; Henderson et al., '42). It was soon shown by Black, McKibbin, and Elvehjem ('41) and later by others that sulfaguanidine inhibited the intestinal elaboration of additional growth factors required by the rat. The inclusion of sulfasuxidine in a synthetic diet gave rise to a biotin and folic acid deficiency (Nielsen and Elvehjem, '42), and recently it has been shown that an inositol deficiency may be produced by feeding sulfasuxidine, Nielsen and Black ('44). However, the rat can synthesize sufficient biotin, folic acid and inositol to meet its requirements of these factors if maintained on a synthetic diet devoid of a sulfonamide. It has been shown by Woolley ('40b) that the mouse requires inositol for normal hair formation. We now have evidence that the mouse, unlike the rat, requires biotin and folic acid for normal growth on an artificial ration.

EXPERIMENTAL

The basal ration had the following percentage composition: alcohol extracted sucrose, 68; alcohol extracted casein, 20; salts IV, 5; corn oil, 5; and cod liver oil, 2. The following B vitamins were incorporated into 100 gm. of the basal diet: thiamine chloride, 0.5 mg.; riboflavin, 1.25 mg.; pyridoxine, 0.5 mg.; calcium pantothenate, 2.2 mg.; nicotinic acid, 5 mg.; choline chloride, 200 mg.; and inositol, 200 mg. The rations were never more than a week old and were stored in an ice box. A special feed cup was used in order to overcome the tendency of mice to scatter the diets. An ointment jar 2 inches in diameter and approximately 2 inches in depth was used as a food container. The jar was filled with ration to within a $\frac{1}{2}$ inch of the top and a metal disc $1\frac{3}{4}$ inches

in diameter was placed over the feed. The disc had three holes of ½ inch in diameter through which all the food had to be obtained by the mice. The jar was fitted with a metal screw top with an opening of 1 inch in diameter, which prevented the removal of the disc by the mice.

Male albino mice supplied by the Rockland Farms were used in this study. They were 21 days old and weighed from 7–9 gm. when placed on the experimental diets. They were divided into groups of eight and placed in cages with raised screen bottoms. The animals were weighed individually and as a group each week.

The basal ration described above did not support good growth in our mice (table 1). The weights reached a plateau at the end of the fourth week on this diet and many of the typical symptoms of a mild biotin deficiency were noted in the animals. The rough fur coat and

TABLE 1

Growth response.

RATION	BODY WEIGHT (AVERAGE OF 8 MICE) AFTER			
	2 Weeks	4 Weeks	6 Weeks	8 Weeks
Basal	20	20	20	23
Basal + Biotin	20	24.5	23	25
Basal + Folic acid	21	25.5	26	28.5
Basal + Biotin + Folic acid	21	27	29	32
Basal + Sulfasuxidine	16	14	Dead	..
Basal + Sulfasuxidine + Biotin	17	19	16.5	Dead
Basal + Sulfasuxidine + Folic acid	18	21	22	20

curvature of the spine are shown in picture A which was taken after the mice had been on this diet 4½ weeks. In another experiment biotin was added to the basal diet at a level of 8 μg. per 100 gm. of ration. The general appearance of the animals was much improved and the animals grew better. A mouse which received the basal ration plus biotin is shown in picture B.

A superfiltrol eluate (eluate factor or folic acid concentrate) was prepared according to the procedure of Hutchings et al. ('41). This concentrate was added to the basal ration at the equivalent of 4 gm. of solubilized liver per 100 gm. of ration. A group of mice receiving this supplement grew better than the animals receiving the basal ration (table 1). The difference in weight indicates that the mouse requires a source of folic acid when maintained on a completely artificial diet.

Another group of mice was given the basal diet to which had been added 8 μg. biotin and a folic acid concentrate the equivalent of 4 gm.

of solubilized liver per 100 gm. of ration. The general appearance of the mice was very good as is seen in picture C, which was taken after the mice had been on this diet 4½ weeks. This group of mice showed greater gains in weight than the groups receiving either supplement alone as we were dealing with a complicated deficiency in the previous experiments. The animals were kept on this ration for 17 weeks and appeared normal in all respects. The individual weight of the animals at this time varied from 34–37 gm.

Since it was believed that mice can synthesize small amounts of biotin and folic acid, sulfasuxidine was incorporated in the basal diet to the extent of 0.6 gm. per 100 gm. of ration. A group of mice receiving this modified diet began to lose weight after the third week and all the animals were dead at the end of 6 weeks. An uncomplicated biotin deficiency was produced by supplementing this ration containing the drug with a folic acid concentrate. This diet supplemented with folic acid gave better growth responses but a typical alopecia developed after 4 weeks. The alopecia was well defined and is seen in picture D. It has been possible to cure and prevent this alopecia with biotin and definite growth of hair was seen after 2 weeks treatment. In another series of mice a severe folic acid deficiency was produced by feeding the basal diet containing the drug together with biotin at 8 μg. per 100 gm. of ration. No alopecia was seen in these animals although the hair coat was untidy. The administration of a folic acid concentrate restored the mice to apparent normal nutrition.

DISCUSSION

The supplementation of the basal ration with biotin and a folic acid concentrate gave a definite growth response. While rats maintained on a similar basal ration can synthesize adequate amounts of biotin and folic acid, it would appear that the bacteria in the intestines of the mouse are unable to meet the full requirements for this species. Since only a mild biotin and folic acid deficiency was produced on the basal ration it is believed that small amounts of these vitamins were synthesized in the intestinal tract but not in adequate amounts for normal growth. A severe biotin deficiency in our animals resulted in a well defined alopecia. A similar alopecia which responds to inositol has been described by Woolley ('40 a, b). Additional work is now in progress to ascertain if biotin is involved in the utilization or absorption of inositol or both.

SUMMARY

1. Biotin and folic acid are required by mice maintained on a completely artificial diet.

2. A severe biotin deficiency in mice is characterized by alopecia.

3. Biotin and folic acid deficiencies produced in mice on a synthetic ration are more acute when 0.6% sulfasuxidine is included in this ration.

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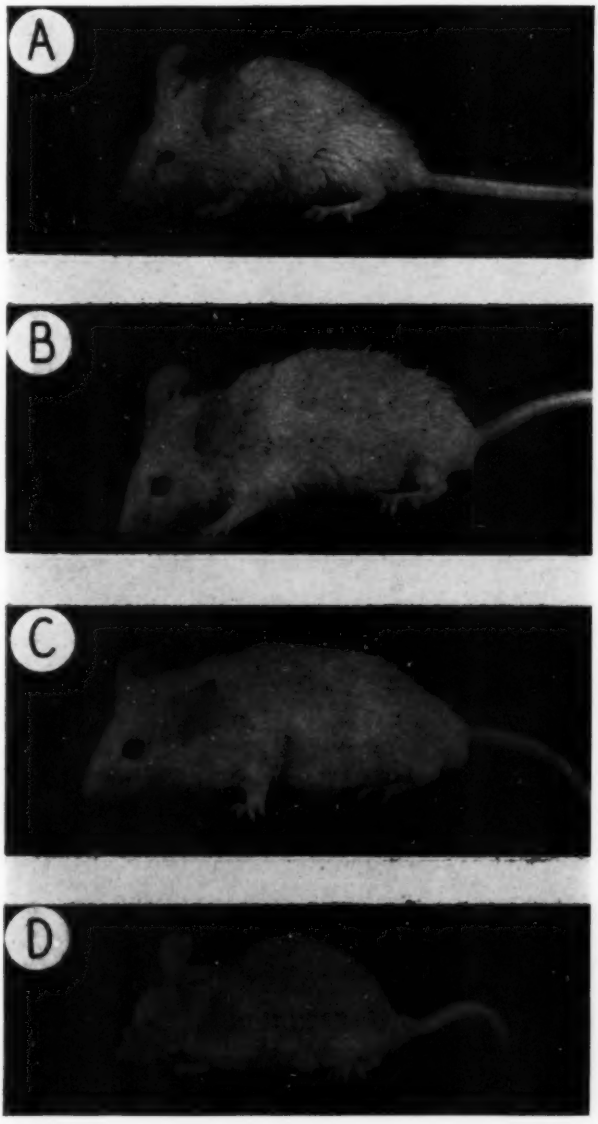

A A mouse which received the basal ration.
B A mouse which received the basal ration plus biotin.
C A mouse which received the basal ration plus biotin and a folic acid concentrate.
D A severe biotin deficiency in a mouse characterized by a well defined alopecia.

DIGESTIBILITY AND BIOLOGICAL VALUE OF SOYBEAN PROTEIN IN WHOLE SOYBEANS, SOYBEAN FLOUR, AND SOYBEAN MILK [1, 2]

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(Received for publication April 15, 1944)

The production of soybeans in this country has greatly increased within recent years. While only 29,000,000 bushels were produced in 1936, the yearly crop had increased to nearly 80,000,000 bushels by 1940, and the goal for 1943 was 216,000,000 bushels (Gortner and Gunderson, '44). Although the bulk of the domestic crop is not destined for human consumption, an important though minor fraction makes its contribution to the human dietary. It has been reported in this connection that 35,000,000 bushels of the 1943 crop would be available in the form of low-fat or high-fat soya flour for consumption by our own populace and by recipients of Lend-Lease (Gortner and Gunderson, '44).

Soybeans are exceptionally rich in protein [3] and it is of considerable practical importance to have as much information as possible on the nutritive qualities of this constituent of the bean. Provided it is of satisfactory nutritional value, the protein in soya products could afford a comparatively inexpensive supplement to the more common sources of food protein.

Experiments with animals have indicated that the protein of whole soybeans, after an appropriate heat treatment, possesses good nutritional qualities and is superior to the proteins of other legumes in its growth-promoting properties (Everson and Heckert, '44). Animal experimentation has also uncovered one of the limitations of the nutritive quality of soybean protein for it has been found that the addition of cystine or methionine improves the utilization by rats and chicks of the protein of both raw and cooked beans (Hayward and

[1] Supported by a grant from the Nutrition Foundation, Inc

[2] Presented at the meeting of the Michigan Academy of Science, Arts and Letters, Ann Arbor, March 17, 1944

[3] The beans used in the present investigation contained about 34% on the basis of N × 5.71.

Hafner, '41). Although these studies with animals are invaluable and have a bearing on human as well as on animal nutrition, experiments with human beings are also desirable. Research of this type is rather costly and requires the painstaking cooperation of a number of individuals. The results of such investigations, however, have the advantage over those obtained in animal studies of being more directly applicable to problems in human nutrition. There is a scarcity of reports of studies with human subjects on the nutritive value of the protein of soybeans. Most of the recorded investigations have dealt with the growth-promoting qualities of milk substitutes containing soybean protein for infants. Only one study on the biological value of soya protein appears to have been carried out with adult human subjects. Cheng, Li, and Lan ('41) reported that the biological value of the protein in soybean curd (protein content of diet 2.6%) was 64%. This value was based on data secured from one 3-day collection period on three subjects for both the experimental soya curd diet and a low-nitrogen basal diet.

The present research was undertaken to study the digestibility and the biological value of soybean protein in adult human subjects. The products examined were whole soybeans [4], soyflour [5], and soymilk.[6]

EXPERIMENTAL

The method employed was that used by Murlin and his associates ('41) to obtain comparable information on the protein in different breads. This procedure involves a comparison of the amount of urinary and fecal nitrogen excreted by subjects fed an especially designed diet containing egg protein, with the amount of nitrogen excreted by the same individuals when fed a similar diet containing the protein to be tested in place of the egg protein. The diet was planned to supply approximately four-fifths of the food nitrogen from either whole egg or the product under investigation, in adjacent test periods. Approximately one-half of the remaining dietary nitrogen was contained in cream, and the rest in the form of fruit, vegetables, and accessories. The total protein level of the diet was maintained at or near 5%

[4] Run-of-the-mill field-grown soybeans were used rather than a single variety. Studies with purified soybean protein from five varieties have shown little, if any, difference in the utilization between the varieties by the rat (G. Everson, personal communication). The soybeans were obtained through the courtesy of Dr. Percy Julian of the Glidden Company, Chicago.

[5] Staley's Soyflour no. 2 was used. It was secured through the courtesy of the A. E. Staley Manufacturing Company, Peoria.

[6] The soybean milk, "Mull-Soy," was generously provided by The Borden Company.

of the total caloric intake and the ratio of carbohydrate to fat was kept constant. Two of the diets eaten in the present study by one of the experimental subjects (F. G.) are shown in table 1. In calculating the biological value of the protein under study, Murlin and his associates

TABLE 1

Sample diets (F.G.. diet squad II).

STANDARD EGG DIET — PERIOD II				
Foodstuff		Calories		
		Prot.	Carbo.	Fat
	gm.			
Egg [1]	246	130.2	..	241
Cream	160	12.7	25.4	535
Starch cracker	2X [4]	2.3	451.2	124
Lettuce	60	2.0	5.7	1.2
Salad dressing	31	..	..	284
Vitamin suppl.	0.25	0.3	..	..
Orange juice	150	4.1	65.0	2.8
Margarine	40	1.2	..	305
Dextri-maltose	172	3.1	670	..
Apple	300	0.2	171	11.1
Sucrose	32	..	128	..
		156.1	1516.3	1504.1

Total calories, 3176.7

Protein, 4.91%
Carbohydrate, 47.7%
Fat 47.3%

WHOLE SOYBEAN DIET — PERIOD III				
Foodstuff		Calories		
		Prot.	Carbo.	Fat
	gm.			
Cooked soybeans	94	131.8 [2]	45.6 [3]	184
Cream	160	12.7	25.4	535
Starch cracker	2X [4]	2.3	451.2	124
Lettuce	60	2.0	5.7	1.2
Salad dressing	37	..	..	342
Vitamin suppl.	0.25	0.3	..	..
Orange juice	150	4.1	65.0	2.8
Margarine	40	1.2	..	305
Dextri-maltose	172	3.1	670.0	..
Apple	300	0.2	171	11.1
Sucrose	20	..	80.0	..
		157.7	1513.9	1505.1

Total calories, 3176.7

Protein, 4.96%
Carbohydrate, 47.7%
Fat 47.3%

[1] Eaten as whole egg omelet.

[2] The percentage of protein in soybeans for use in the calculation of the protein calories was obtained by multiplying the percentage of soybean nitrogen by the factor 5.71, which is the nitrogen factor given by Jones ('31).

[3] The soybean carbohydrate calories were based on the percentage of available carbohydrate. Adolph and Kao ('33) have reported that 40% of soybean carbohydrate is utilized by the animal body.

[4] The starch cracker was eaten to supply calories with little nitrogen. It was patterned after the cracker employed by Sumner and Murlin ('38) in one of their studies. The batter for the cracker used in the present investigation consisted of corn starch, 600 gm., gum acacia 32 gm., Crisco 80 gm., alum baking powder 20 gm., Karo 80 gm., salt 8 gm., and water 800 gm. 270 gm. of this batter poured into two pie tins and baked slowly for 1 hour provided two starch crackers. Two such crackers were eaten by each subject.

('41) made the tacit assumption that the egg protein was completely utilized. The calculated biological value was based, therefore, on egg protein as 100.

The experimental subjects were all males from the Medical School staff and student body. Owing to the nature of the research, only a limited number of individuals could be studied together. Experiments were carried out with three separate diet squads, one squad being studied at a time. Data on the ages, heights, body weights, and the distribution of calories in the experimental diets for each subject in the different diet squads, together with dates showing the duration of the experiments with each squad, are given in table 2.

The subjects ate all of their meals in one of the departmental laboratories which served both as a dining room and kitchen. During a preliminary interval the individuals were fed a standard egg diet for a period of several days (6 to 10 days), during which time adjustments were made in the diet and the subjects became familiar with the routine of eating together and of collecting feces and urine. After the preliminary interval, the first soybean product period began. This was followed by egg and soya product periods, so spaced that a soya product period was always adjacent to an egg period. All of the experimental periods were 6 days in length.

The subjects remained in good health throughout the study and the data in table 2 show that changes in body weight were only moderate. The diet promoted normal gastrointestinal function in all of the subjects. More frequent defecation and better-formed stools were observed during each of the soybean product periods than during the standard egg diet periods. The high satiety value of the ingested soybean products was noted.

The soybean products employed as the source of the bulk of the nitrogen in the different experimental diets were prepared for consumption as described below. The whole soybeans were autoclaved with 2 parts of water at 15 pounds pressure for 1 hour. This treatment rendered them comparable in softness and flavor to soybeans cooked for several hours at normal atmospheric pressure. They were reheated for serving. The soyflour was mixed with water and salt (2½ oz. flour, ¾ cup water, and ¼ teaspoon salt) and autoclaved for 1 hour. Individual portions of the soya products were cooked daily. Although the soymilk was not autoclaved, heat had been employed in the course of its manufacture. The soymilk was warmed before drinking. Whole egg was used in the form of an omelet. The eggs, secured from one local source, did not vary appreciably in protein con-

TABLE 2

The diet squads[a] and the caloric distribution in the experimental diets.

Date	SQUAD	AGE	HEIGHT	BEGINNING WEIGHT	WEIGHT CHANGE	CALORIES PER DIEM			
						Total intake	Distribution		
							Prot.	Carbo.	Fat
		yrs.	*cm.*	*kg.*	*kg.*		%	%	%
Nov. 16 — Dec. 22, '42	I								
	W. C.	31	178.0	67.6	— 1.8	2607 [b]	5.0	47.5	47.5
						2594 [c]	4.7	47.6	47.7
						2605 [d]	4.9	47.5	47.6
	S. K.	24	180.2	75.5	+ 0.5	3607 [b]	5.0	47.5	47.5
						3593 [c]	4.7	47.6	47.7
						3607 [d]	5.0	47.4	47.6
	K. P.	22	190.6	67.0	+ 0.7	3607 [b]	5.0	47.5	47.5
						3593 [c]	4.7	47.6	47.7
						3607 [d]	5.0	47.4	47.6
	R. R.	22	171.2	62.5	— 0.7	2607 [b]	5.0	47.5	47.5
						2594 [c]	4.7	47.6	47.7
						2605 [d]	4.9	47.5	47.6
Feb. 2 — Mar. 16, '43	II								
	R. C.	22	184.2	86.7	— 0.2	3799 [b]	4.9	47.6	47.5
						3805 [c]	4.9	47.5	47.6
						3804 [d]	4.9	47.6	47.5
	J. G.	23	165.1	64.5	0.0	2995 [b]	5.1	47.5	47.4
						2987 [c]	5.1	47.5	47.4
	F. G.	21	178.0	61.0	+ 0.1	3177 [b]	4.9	47.7	47.3
						3177 [c]	5.0	47.7	47.3
						3179 [d]	5.0	47.4	47.6
	J. H.	22	170.9	67.5	+ 0.5	3208 [b]	4.9	47.7	47.4
						3206 [c]	5.0	47.5	47.5
						3208 [d]	4.9	47.5	47.6
	G. V.	24	174.9	90.4	— 2.2	3167 [b]	4.9	47.6	47.5
						3168 [c]	4.9	47.6	47.5
						3168 [d]	4.8	47.7	47.5
	J. Y.	22	183.5	74.0	+ 0.5	3500 [b]	5.0	47.5	47.5
						3504 [c]	5.0	47.5	47.5
						3503 [d]	5.0	47.5	47.5
May 24 — June 22, '43	III								
	R. C.	22	184.2	86.3	0.0	3798 [b]	4.9	47.6	47.5
						3797 [e]	4.8	47.6	47.6
	B. C.	22	181.4	77.5	— 0.5	3584 [b]	4.9	47.6	47.5
						3597 [e]	5.0	47.5	47.5
	A. F.	22	180.0	71.4	— 1.6	3302 [b]	5.0	47.6	47.4
						3299 [e]	4.9	47.6	47.5
	F. G.	21	178.0	62.1	0.0	3190 [b]	4.9	47.6	47.5
						3190 [e]	4.9	47.6	47.5
	J. H.	22	170.9	68.7	+ 0.5	3207 [b]	4.9	47.6	47.5
						3209 [e]	4.9	47.6	47.5
	S. Z.	23	169.0	63.1	+ 1.6	2989 [b]	5.1	47.5	47.4
						2985 [e]	5.0	47.5	47.5

[a] Three of the subjects served on two diet squads; all others served only once.
[b] Standard egg diet containing whole egg omelet as the chief source of protein.
[c] Diet containing autoclaved whole soybeans as the chief source of protein.
[d] Diet containing soybean "milk" as the chief source of protein.
[e] Diet containing autoclaved soybean flour as the chief source of protein.

tent as determined by nitrogen analyses. One brand of oleomargarine was employed throughout the study; it was much lower in nitrogen than butter. The cream (36%) was secured by special arrangement from a local dairy. The orange juice was prepared from California Valencias or Florida oranges, depending on the season; the apples were largely Northern Spies. Dextrimaltose [7] and sucrose were used to augment the carbohydrate calories. Salad dressing was always made from the same brands of commercial salad oil and distilled vinegar. One vitamin capsule [8] daily provided 1.5 mg. of thiamine chloride, 2 mg. of riboflavin, 1 mg. of calcium pantothenate, 10 mg. of niacin, 250 μg. of pyridoxin, and yeast and liver extract. The gelatin shell of the capsule was not consumed with the vitamins.

All analyses for nitrogen were made by the macro Kjeldahl method. Foods were sampled periodically and nitrogen determinations made on aliquots of pooled samples. Urine was analyzed daily for the last 4 days of each period. Nitrogen analyses were made on the fecal material collected during each 6-day period. Charcoal markers taken with the first meal of each period permitted separation of the feces of one period from those of another. The fresh feces were kept covered with ethanol containing 1% sulfuric acid until dried on the steam cone. Nitrogen determinations were made on aliquots of the ground dried material.

For purposes of calculation of the biological value, the average daily nitrogen excretion in the feces and in the urine by subjects fed a soybean product diet was compared to that of the same subjects fed the comparable standard egg diet, according to the method of Murlin and his associates ('41), details of which are shown in table 3. In most cases the daily averages were obtained from the data for two periods for each type of diet. In the experiment with diet squad II, for instance, diets were eaten in the following order: preliminary egg, whole soybean, standard egg, whole soybean, soymilk, standard egg, soymilk. The daily average nitrogen excretion during the two whole soybean periods was compared in the prescribed manner with that of the two standard egg periods; the daily average nitrogen excretion during the two soymilk periods was similarly compared. The urinary excretion of nitrogen during the two standard egg periods was not identical — sometimes a few decigrams less nitrogen was excreted in the second period by a given individual than in the first, sometimes

[7] We are indebted to Mead Johnson and Company for the Dextrimaltose no. 1 used in these experiments.

[8] Kindly provided by the Gelatin Products Company.

TABLE 3

TABLE 3

Digestibility and biological value of soybean protein in cooked whole soybeans, cooked soybean flour and soybean "milk" (based on daily averages).

TYPE OF SOY FOOD	SUBJECT	*a* SOY FECAL N	*b* EGG FECAL N	*c* (a-b) FOOD FECAL N	*d* TOTAL FOOD N	*e* (d-c) ABSORBED N	*f* TRUE DIGESTIBILITY $\frac{\text{ABS. N} \times 100}{\text{TOTAL FOOD N}}$	*g* SOY URINE N	*h* EGG URINE N	*i* (g-h) "EXCESS URINE N"	*j* $\frac{\text{"EXCESS N"}}{\text{ABSORBED N}} \times 100$	*k* B. V. (100-j)
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	%	*gm.*	*gm.*	*gm.*	%	%
Soybeans	W. C.	1.143	0.846	0.297	5.099	4.802	94.2	4.836	4.494	0.342	7.1	92.9
	R. C.	2.093	1.108	0.985	7.877	6.892	87.5	6.012	5.883	0.129	1.9	98.1
	J. G.	1.409	0.857	0.552	6.397	5.845	91.4	4.852	4.169	0.683	11.7	88.3
	F. G.	1.793	1.208	0.585	6.713	6.128	91.1	4.985	4.332	0.653	10.7	89.3
	J. H.	2.006	1.043	0.963	6.713	5.750	85.7	4.676	4.955	−0.279	− 4.9	104.9
	S. K.	1.747	1.083	0.664	7.022	6.358	90.5	4.596	4.360	0.236	3.7	96.3
	K.P.	1.763	0.995	0.768	7.022	6.254	89.1	4.524	4.678	−0.154	− 2.5	102.5
	R. R.	1.083	0.977	0.106	5.099	4.993	97.9	4.604	3.826	0.778	15.6	84.4
	G. V.	1.530	0.925	0.605	6.493	5.888	90.7	6.146	5.881	0.265	4.5	95.5
	J. Y.	2.027	1.012	1.015	7.451	6.436	86.4	5.646	5.188	0.458	7.1	92.9
							(90.5 av.)					(94.5 av.)
Soyflour	R. C.	1.789	1.287	0.502	7.692	7.190	93.5	5.445	5.018	0.427	5.9	94.1
	B. C.	1.969	1.339	0.630	7.483	6.853	91.6	4.892	4.304	0.588	8.6	91.4
	A. F.	1.419	1.127	0.292	6.815	6.523	95.7	4.877	4.522	0.355	5.4	94.6
	F. G.	1.458	1.188	0.270	6.615	6.345	95.9	4.133	3.810	0.323	5.1	94.9
	J. H.	1.678	1.158	0.520	6.615	6.095	92.1	5.120	4.300	0.820	13.5	86.5
	S. Z.	1.609	1.286	0.323	6.312	5.989	94.9	4.460	3.788	0.672	11.2	88.8
							(94.0 av.)					(91.7. av.)
Soymilk	W. C.	1.418	0.846	0.572	5.409	4.837	89.4	4.418	4.494	−0.076	− 1.6	101.6
	R. C.	1.948	1.108	0.840	7.772	6.932	89.2	6.203	5.883	0.320	4.6	95.4
	F. G.	1.836	1.208	0.628	6.658	6.030	90.6	4.764	4.332	0.432	7.2	92.8
	J. H.	1.976	1.043	0.933	6.659	5.726	86.0	5.003	4.955	0.048	0.8	99.2
	S. K.	1.658	1.083	0.575	7.514	6.939	92.4	5.622	4.360	1.262	18.2	81.8
	K. P.	1.849	0.995	0.854	7.514	6.660	88.6	5.479	4.678	0.801	12.0	88.0
	R. R.	1.122	0.977	0.145	5.409	5.264	97.3	3,880	3.826	0.054	1.0	99.0
	G. V.	1.754	0.925	0.829	6.397	5.568	87.0	5.604	5.881	−0.277	− 5.0	105.0
	J. Y.	2.079	1.012	1.067	7.379	6.312	85.6	5.492	5.188	0.304	4.8	95.2
							(89.6 av.)					(95.3 av.)

vice versa. In some instances data were available for certain subjects for only one period in which a certain type of diet was eaten. Thus, in the first diet squad experiment four individuals ate whole soybeans for one period instead of the usual two and the daily averages for this one food product were based on one period for these subjects. Similarly, the subjects of diet squad III ate the standard egg diet for only one period owing to the untimely interruption of the experiment by the Detroit race riot.

DISCUSSION OF RESULTS

The values for the true digestibility and for the biological value of soya products tested are given in table 3 for each of the experimental subjects. The data in table 3 show little variation in the average protein digestibility between the soybean products used in this study. The average values for autoclaved whole soybeans, autoclaved soyflour, and soymilk are 90.5, 94.0, and 89.6%, respectively. For purposes of comparison it may be mentioned that Tso and Chu ('31), in studies with infants, recorded values averaging 80% when soya protein was fed in the form of soybean "milk."

The average biological values for maintenance for the three soya products are not widely dissimilar, being 94.5% for the protein in cooked whole soybeans, 91.7% for cooked soybean flour, and 95.3% for soybean "milk." It is noteworthy for purposes of comparison that the biological values for maintenance of the protein of a wheat cereal biscuit, a whole wheat bread, and a white bread as determined by the method employed in the present study were 81.6%, 77.8%, and 74.6%, respectively (Murlin, Marshall, and Kochakian, '41).

The comparatively high biological value of soybean protein as determined in our studies with human subjects is in accord with the the results of investigations with animals which have given evidence of the good nutritional qualities of the protein of this legume. Not only is suitably prepared soybean protein capable, as the sole source of protein in the diet, of supporting good growth in experimental animals, but soybean flour supplements greatly improve the nutritive value of patent flour (Jones and Devine, '42).

It is well known that the biological value of dried uncooked soybean protein may be improved by a certain degree of heating (Johnson, Parsons, and Steenbock, '39). No attempt was made in the present study to assess the effect of heat on the biological value of protein in the soybean products eaten. The whole soybeans and the soybean

flour used in our experiments were cooked (autoclaved) for the practical purpose of making them palatable.

The nitrogen balances calculated for the different experimental periods indicated that the level of protein in the experimental diets was well-suited to bring out differences between the egg protein and the protein of the various soybean products studied. The average daily nitrogen balance for all of the egg diet periods was + 0.787 gm., while the comparable balance values for the whole soybean, soyflour, and soymilk diets were — 0.147, + 0.298, and — 0.162 gm., respectively. As shown in table 3, the subjects excreted more nitrogen in the feces when fed the soya product diets than when fed the standard egg diet. This finding was in part responsible for the less favorable nitrogen balances observed when soybean diets were consumed. Rose and MacLeod ('25) observed that the "cost of digestion," as assessed by the excretion of fecal nitrogen, was greater for soybean curd than for the protein in meat, and in bread and milk.

Both the digestibility and the biological value of a protein are important, of course, in determining its over-all nutritive value. By multiplying the digestibility by the biological value a quantitative expression is obtained which might be termed the nutritive index. The comparative nutritive index of the protein in whole cooked soybeans, as determined in the present maintenance experiments, is 0.9 multiplied by 94.5%, or 85 compared to egg taken as 100.[9] Although the exigencies of digestion and consequent loss of nitrogen in the feces reduce somewhat the gross nutritive value of the soya protein, the soybean appears to be a valuable adjunct to the more common sources of dietary protein.

SUMMARY

The average true digestibility in adult human subjects of the protein in cooked whole soybeans, in cooked soybean flour, and in soybean milk was found to be 90.5%, 94.0%, and 89.6%, respectively.

The average biological value of soybean protein for maintenance in adult human subjects, as determined by the method of Murlin et al. ('41) in which the protein of whole egg is employed as a standard, was found to be 94.5% for the protein in cooked whole soybeans, 91.7% for that in cooked soyflour, and 95.3% for that in a commercial soybean "milk."

[9] A value of 100 would presuppose not only complete digestibility of egg protein but also a biological value of 100%. Actually, the average biological value of the protein of whole egg, as determined in experiments with young rats (Mitchell and Carmen, '26) is 94%. The true nutritive index of whole egg protein, as well as that of soybean protein, is, therefore, somewhat lower than the value given in the text.

Under the conditions of the present study less favorable nitrogen balances were observed when the experimental subjects were fed diets containing soybean products than when they were fed a standard egg diet, owing largely to greater loss of soybean nitrogen in the feces.

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